

OPTIMIZATION OF A SEROLOGICAL ASSAY SYSTEM FOR ESTABLISHING
INFECTION OF KAPOSI'S SARCOMA ASSOCIATED HERPESVIRUS (KSHV) IN
MULTIPLE POPULATIONS AT VARYING LEVELS OF RISK

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Background: Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8) is the etiologic agent of Kaposi's sarcoma (KS), multicentric Castleman's disease (MCD), and primary effusion lymphoma (PEL). KSHV is a nonubiquitous herpesvirus (~3% in the general US population) that can cause significant morbidity and mortality among immunocompromised hosts. However, routine surveillance of KSHV is lacking because diagnostic systems for viral identification are not of high enough sensitivity and specificity. This research describes a novel KSHV serological algorithm which increases sensitivity and specificity of KSHV detection beyond what has been previously reported.

Methods: A novel KSHV assay algorithm based on a baculovirus-expressed LANA1-GST fusion protein was used with previously described KSHV lytic antigen ELISAs. Initial assay evaluation was performed using 90 case sera from persons with AIDS- KS and 100 blood donor controls. We identified two multi-antigen algorithms: one that maximized sensitivity and one that maximized specificity. Sera from patients requiring bone-marrow transplantation, cadaveric renal transplant donors (CRTD), patients with systemic lupus erythematosus (SLE) and subjects with primary (PPH) and secondary pulmonary hypertension were obtained for KSHV testing.

Results: The highly sensitive algorithm yielded a sensitivity of 96% and a specificity of 94% and the highly specific algorithm a sensitivity of 93% and a specificity of 98%.

Among CRTD, using the highly specific algorithm, overall seroprevalence was low at 4.0% (2/50) and similar to blood donors ($P=0.46$; $OR=1.4$; $CI=0.14, 7.9$). With the more specific algorithm, 8.0% (4/50) were infected compared to 6.4% (16/250) among blood donors ($OR=1.3$; $CI=0.41, 4.0$; $P=0.43$). Among subjects requiring bone marrow transplantation seroprevalence was 3.0% and 10.0% and did not differ from blood donors ($OR=2.0$; 95% $CI=0.10, 122.9$; $P=0.50$). Higher KSHV seroprevalence was observed among SLE patients using the specific algorithm ($OR=6.0$; 95% $CI, 1.2-29.0$) and the sensitive algorithm ($OR=3.6$; 95% $CI, 1.1-12.2$) though this is likely due to antigenic cross-reactivity as opposed to actual infection. Among patients with PPH we found no evidence of KSHV infection (0/19).

Conclusions: We used a systematic approach to standardize the assessment of KSHV infection rates and examined seroprevalence rates among high-risk populations of clinical interest. KSHV is of public health importance because it leads to cancer in immunocompromised hosts. Future studies of KSHV should focus on the cost-effectiveness of implementing surveillance systems such as the one described here, which could potentially lead to a marked reduction in KSHV-associated morbidity and mortality.

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1. Kaposi's sarcoma-associated herpesvirus: Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is the most recently described human herpesvirus. Discovered by Chang and Moore in 1994(100, 298), KSHV is a nonubiquitous herpesvirus which most often causes clinical manifestations among immunocompromised hosts. In fact, early in the AIDS epidemic of the 1980's, Kaposi's sarcoma (KS), caused by KSHV, was the most common AIDS defining illness.

Though the morbidity and mortality associated with KSHV infection among immunocompromised hosts is substantial, routine surveillance of infection among these populations is lacking. Currently, diagnostic systems for identifying KSHV infection are not of high enough sensitivity and specificity to be used in the clinical setting. The following chapters describe the epidemiology and public health impact of KSHV infection among immunocompromised and immunocompetent hosts, as well as research describing a new algorithm for defining KSHV infection using established and novel serological assays. In conclusion, the potential impact of the research is addressed with specific attention being paid to public health and epidemiology.

1.1. Clinical Manifestations

KSHV is known to be associated with three conditions; Kaposi's sarcoma (KS), a blood vessel cancer, multicentric Castleman's disease (MCD), a form of severe lymph node enlargement and primary effusion lymphoma (PEL), a cancer of the lymphocytes(295, 373). In addition, case series suggest that KSHV may play a role in a newly described syndrome of bone marrow failure after transplantation(121, 258). Of these, KSHV is suspected as the causal agent in each, though current evidence supports a

definitive causal role only with KS. Though other disease associations have been sought, no conclusive evidence exists to support a consistent relation between KSHV and any other disease or syndrome at this time. Scientists have not ruled out KSHV as a co-factor in other disorders however, and many groups are actively pursuing other diseases which may be associated with the virus.

Of the diseases caused by KSHV, KS is most clinically important with an estimated annual incidence of 5 cases per 100,000 per year(307). In the United States, KS is most common among homosexual men with AIDS. However, due to improvements in AIDS therapies, KS incidence has been in decline among this population. Historical data suggests that KS incidence is not in decline among other populations however, and in fact may be more common today than it was just 50 years ago.

The current dogma is that among individuals without underlying immunosuppression KSHV is largely asymptomatic. In a case-control study of HIV-negative males, some acute non-specific symptoms such as diarrhea, fatigue, rash and lymphadenopathy were more common among those recently acquiring KSHV than among non-seroconverters(257, 314). However, in actuality, little information exists as to the actual risk of KS among immunocompetent KSHV infected individuals(142). Long term follow-up studies conducted in such a population with appropriate matched controls have not been undertaken. Additionally, cancer statistics data bases, such as the Surveillance Epidemiology and End Results (SEER) data base, do not stratify KS incidence and prevalence data by HIV infection status. Therefore, accurately assessing the rate of KS among HIV uninfected individuals using national surveillance data is not

currently possible. One assumption which has been made is that KS incidence in the US among HIV uninfected individuals has remained constant over the last 50 years and rates prior to the AIDS epidemic are reflective of current rates(43), though the accuracy of such an assumption is difficult to directly examine.

In general, the risk of acute measurable KSHV associated morbidity and mortality among the general population is likely low. However, the long-term consequences of infection have not been explored. Longitudinal studies which track survival, in addition to morbidity assumed to be directly unassociated with KSHV infection, are required to provide a definitive answer to the actual effects of KSHV infection among the general population.

Though KSHV is associated with at least three diseases: Kaposi's sarcoma (KS), body cavity-based lymphoma; and multicentric Castleman's disease, (295, 373). Other disease associations, including multiple myeloma, sarcoidosis, primary pulmonary hypertension(68, 110), and post-transplant skin cancers, have been suggested but these associations have either not been adequately confirmed or later refuted(122, 193, 225, 240). The following sections describe the known clinical syndromes in which KSHV is associated and discusses other diseases in which KSHV is now known not to play a role.

1.1.1. Kaposi's Sarcoma

The most important clinical manifestation of KSHV infection is Kaposi's sarcoma (KS). Described in 1872 by Moritz Kaposi, KS is a neoplastic disorder first identified in five patients as an idiopathic multiple pigmented sarcoma of the skin. Since that time four distinct epidemiologic forms of KS have been defined; classic KS, endemic KS, AIDS-KS, and iatrogenic KS. KSHV is an etiologic co-factor in common to all forms of

KS, and the histological features of KS are indistinguishable between the clinical variants.

KS is a proliferative condition with lesions comprised of spindle cells surrounding vascular slits. There are three distinct histologic stages of KS and include the patch, plaque, and nodular forms. Though KS is most commonly localized to the skin, organ involvement can occur and is not uncommon in AIDS-KS.

1.1.1.1. Classic Kaposi's Sarcoma

Classic KS presents as a disease of the feet and hands primarily among older men of Mediterranean and Jewish descent. The male to female ratio of classic KS has been reported to be 15:1 (18, 133) with age of onset most common at greater than 50 years of age(120, 205, 368, 415) and rarely (4%-8%) prior to 30 years of age(42, 132, 175, 197, 422). The least aggressive form of KS, classic KS is a slow or non progressing disease that when even left untreated rarely invades the visceral compartments(211). The mean survival of patients with classic KS is 10 to 15 years with cause of death generally attributed to an unrelated condition associated with age(106).

Epidemiologically, classic KS has the highest incidence rates in Italy, Greece, and Israel(211) and even prior to the AIDS epidemic, rates of reported KS incidence began to rise. Whether these observations were due to increased reporting, misdiagnosis, or were the result of an actual increase, population-based reporting of KS was notably elevated throughout Europe and Israel in the 1960's and 1970's(132, 197, 211, 212, 422). In contrast, within the US, classic KS is rare with only an approximated 600 cases reported prior to 1950(282). In a retrospective analysis performed by Biggar et al., the annual KS incidence prior to the AIDS epidemic was estimated to be 0.29 cases/100,000/year among

men and 0.07 cases/100,000/year among women(42). Currently in the United States reporting of KS includes all forms, of which AIDS-KS is the majority, therefore accurate assessments of rates of the classic form are unavailable, though it is still believed that classic KS in the Western hemisphere is extraordinarily rare.

1.1.1.2. Endemic Kaposi's Sarcoma

Recognition of KS as a common cancer in parts of Africa became apparent in the literature of the 1950s. Prior to the AIDS epidemic, KS was not uncommon and represented 9%-13% of all malignancies in Zaire and 3%-9% in Uganda(109, 205, 312, 414). Endemic KS has two epidemiologic sub-categories. One form affects young adults, with mean age of onset at 35 years of age and a male to female ratio of approximately 15:1. Survival rates among this group range from 5 to 8 years after diagnosis(410, 429). The second form, which is highly aggressive and associated with high mortality, affects children with mean age of onset at 3 years of age and a male to female ratio of 3:1. KS comprises 2%-10% of all cancers among children in central Africa(23, 105).

Studies of endemic KS are now difficult to perform in the setting of the African AIDS epidemic. KS is currently the most common form of cancer in men and second most common cancer among adult women in much of central Africa. An example of how the HIV epidemic confounds the true assessment of endemic KS can be shown in other changes observed in KS incidence since AIDS. Namely, the male to female ratio has dramatically shifted to near parity with reported male to female ratios observed to be 2:1 in 1996(393). This observation is likely not due to differences in KSHV exposure status, as men and women have similar KSHV prevalence in Africa(281, 316). Some have

hypothesized that hormonal dynamics may play a role in the development of KS though currently no direct evidence exists to support firm conclusions(316).

Though highly active anti-retroviral therapy (HAART) has reduced the incidence of KS in the Western world, incidence of KS in Africa is still common and likely the result of untreated HIV infection(106). As routine HAART therapy becomes more readily available, especially among children, it is assumed that KS prevalence rates will likely begin to decline in Africa. However, with the extraordinarily high rate of HIV and KSHV viral endemicity the problem of KS in Africa will likely not soon be under control.

1.1.1.3. AIDS-associated Kaposi's Sarcoma

Prior to the AIDS epidemic, KS in the US was very rare with an estimated 2 to 6 cases per million individuals per year annual incidence(206, 312, 367, 368). However, in the summer of 1981 large numbers of homosexual men in New York and California began presenting to hospitals with the distinctive skin lesions of KS(96, 167, 206). It soon became apparent that a number of rare opportunistic infections were suddenly of epidemic proportions.

The incidence rate of KS among HIV-infected individuals has been reported to be several thousand folds higher than the general population(211). Among homosexual men with HIV infection at the beginning of the AIDS epidemic the risk of developing KS approached 50%(44, 45), and approximately 40% of patients who received a diagnosis of AIDS presented with KS. More recently, it has been noted that among KSHV/HIV coinfecting males the 10 year risk of KS is 39%-50%(275, 310).

Overall annual KS incidence peaked at 10.2 per 100,000 in white males in 1988 and 10.5 per 100,000 in black males in 1992 (Figure 1-1).

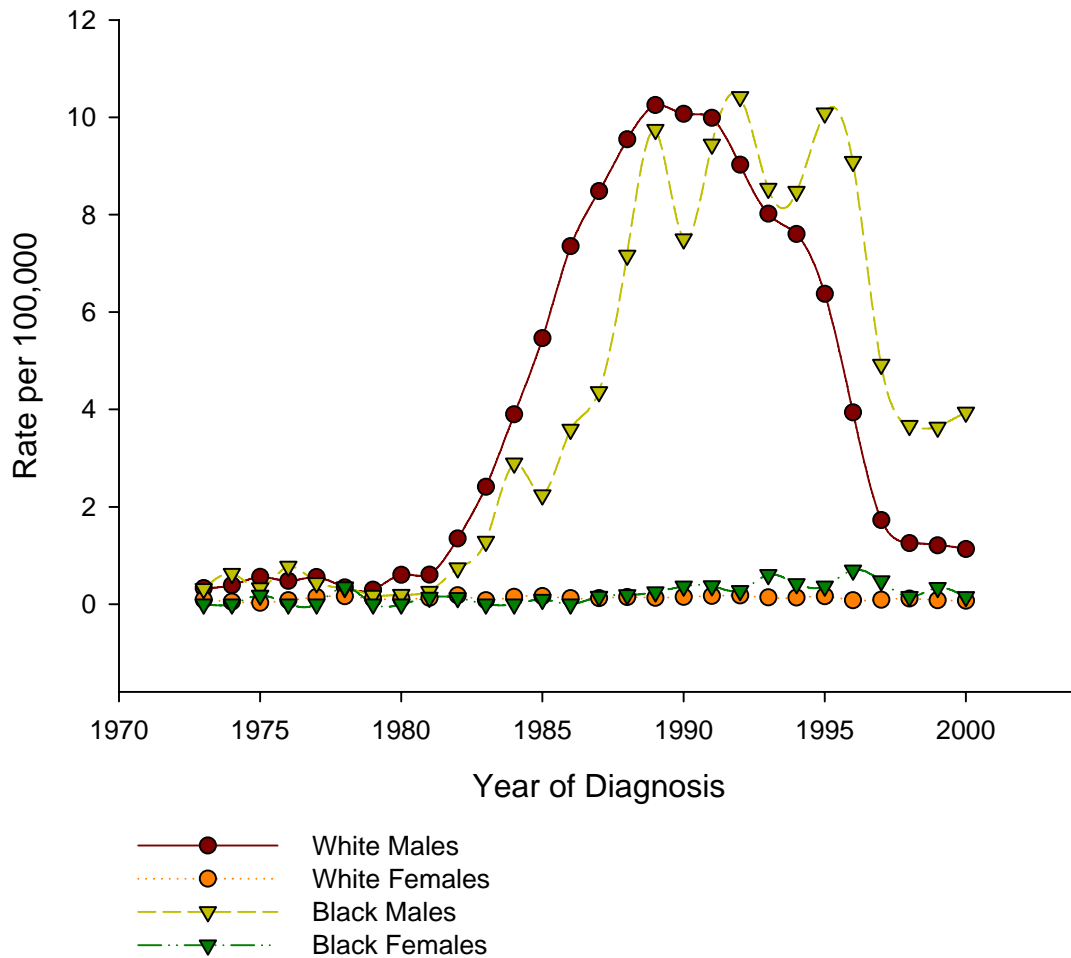


Figure 1 -1. SEER Incidence Age-Adjusted Rates of Kaposi's Sarcoma 1973-2000

Nearly half of HIV-positive males are infected with KSHV, yet, as Figure 1-1 shows, KS rates among this group have fallen dramatically in recent years. Some of this fall can be

attributed to improvements in the overall health of HIV-positive individuals due to HIV-targeted immuno-reconstitution therapies such as HAART, introduced in the mid 1990s(307). Many studies have shown that HAART, particularly a regimen containing at least one protease inhibitor, is effective in reducing skin lesions and prolonging life in AIDS-KS patients(52, 245, 408). Other reasons for the noted decline in KS likely include an expanded definition of AIDS-defining criteria, decreased reporting of minor dermatological cases and increased use of safe-sex practices associated with HIV prevention campaigns. Though KS incidence has steadily declined over the last decade, it is still the most common neoplasm affecting people with HIV today(4, 78).

Table 1-1 presents the most recent rates of KS observed in the US. Overall rates are higher in the southern US compared to the rest of the country. Similar geographical patterns exist for other sexually transmitted infectious diseases. However, this geographical pattern cannot fully account for the increased incidence of KS observed in some locations such as the District of Columbia. A complete understanding of the geographic distribution of KS in the US is not possible at this time though it is likely that reporting practices differ and may account for some of the variability associated with incidence data.

Table 1-1. Age-adjusted invasive Kaposi's Sarcoma incidence rates and 95% confidence intervals by US census region and division, state and metropolitan area, all races.

Area	Male
United States	0.9 (0.8-0.9)
Northeast	0.7 (0.5-1.0)
Massachusetts	0.9 (0.6-1.3)
Middle Atlantic	1.2 (1.1-1.4)
New Jersey	1.0 (0.7-1.4)
New York	1.6 (1.3-1.9)
Pennsylvania	0.8 (0.6-1.1)
Midwest	0.4 (0.4-0.5)
<u>East North Central</u>	0.4 (0.3-0.5)
Michigan	0.5 (0.3-0.8)
Detroit	1.2 (0.7-1.8)
<u>West North Central</u>	0.4 (0.3-0.6)
South	
<u>South Atlantic</u>	
District of Columbia	5.3 (3.0-9.1)
Florida	1.3 (1.0-1.5)
Georgia	1.3 (1.0-1.7)
Atlanta	2.9 (2.1-4.3)
<u>West South Central</u>	0.8 (0.7-1.0)
Louisiana	1.1 (0.7-1.8)
Texas	0.8 (0.6-1.0)
West	1.1 (1.0-1.3)
<u>Mountain</u>	0.6 (0.4-0.8)
<u>Pacific</u>	1.4 (1.2-1.5)
California	1.5 (1.4-1.8)
San Francisco-Oakland	2.8 (2.1-3.7)
Los Angeles	2.3 (1.9-2.8)
Washington	0.9 (0.6-1.4)
Seattle-Puget Sound	1.0 (0.6-1.6)

Note: Rates are per 100,000 and are age-adjusted to 2000 US standard population. Rates cover approximately 92% of the US population. Data from US cancer statistics 2004 report(424).

In addition to the noted decreasing rates of KS, survival rates among patients with KS have increased. In contrast to classic KS, AIDS-KS is highly aggressive and life-threatening visceral and mucosal involvement is common. In a postmortem study by Friedman et al., 77% of patients with AIDS-KS showed signs of visceral involvement at

autopsy(168). Lemlich et al. found, at autopsy, that among AIDS-KS patients with no visible skin lesions that 29% had visceral involvement(248). In the 1980's KS prognosis was poor, though through the use of HAART, survival has dramatically improved to rates approximately half that observed prior to the AIDS epidemic (Figure 1-2)(307).

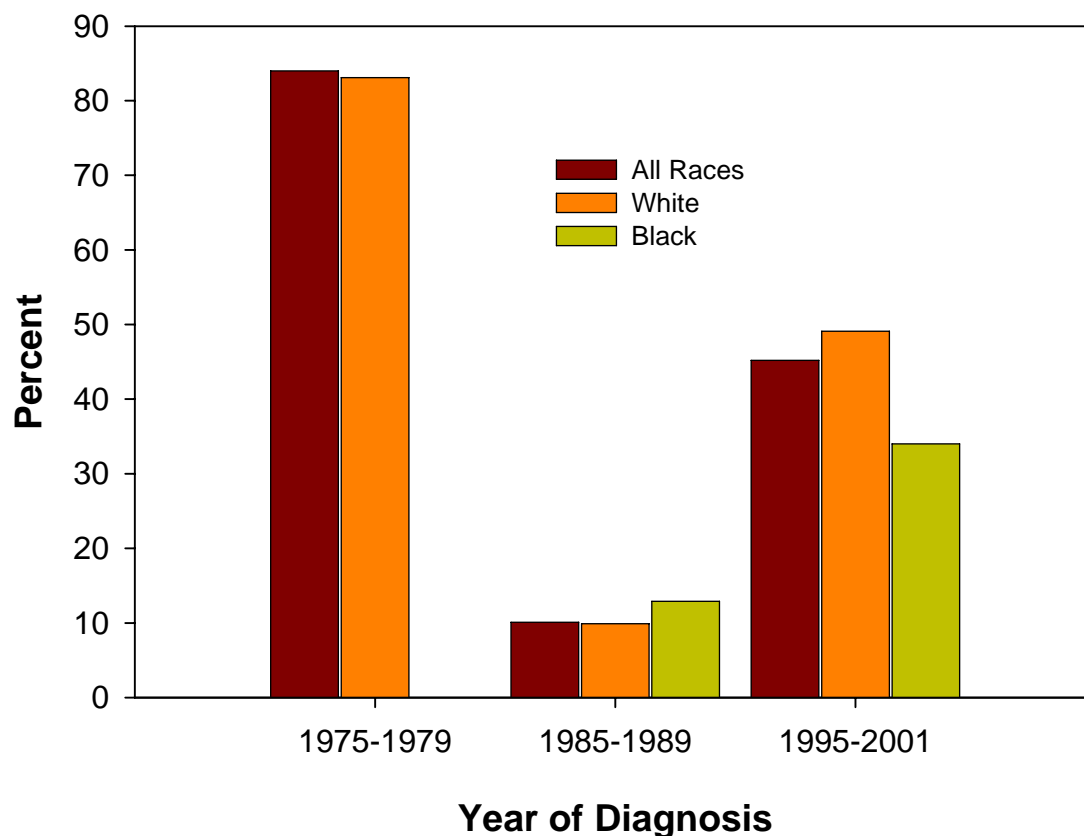


Figure 1-2. SEER 5-Year Kaposi's Sarcoma Relative Survival Rates by Year of Diagnosis Note: Data from National Cancer Institute(363).

Two factors associated with the development and progression of KS are high antilytic KSHV antibody titers and detection of KSHV DNA in peripheral blood(157, 158, 161, 403). Observations in mice models suggest inflammatory cytokines (IC)

trigger a cascade of events that lead to the genesis of KS lesions. Experimental studies suggest that IC upregulation stimulates KSHV reactivation in circulating cells resulting in viral spread and dissemination in tissues(158, 160, 161). KSHV viral dissemination causes an induction of the host immune response, but the full significance of the immune response is currently unknown.

If ongoing viral activity is necessary for KS development, then the KSHV specific antibody response may play an important role in preventing KS. However, because mean titers are usually higher in persons with KS, it is not clear that a KSHV antibody response is protective against KS. Furthermore, in some studies CD4+ and CD8+ T cells and natural killer cells appeared ineffective in clearing KSHV infected cells from circulation(159, 403). If KS development is due to the inability of the immune response to clear viral DNA in circulating cells, then high antibody titers would presumably not be protective against KS disease development and progression. However, these observations may also be the result of an ecological fallacy(239, 241).

Markers used to assess the severity of HIV/AIDS disease have been shown to be useful in assessing KS disease progression. HIV viral load is associated with KSHV viremia(417) and is strongly associated with the severity of KS disease. Lower CD4 lymphocyte counts contribute to increased KS tumor burden and decreased survival times in patients with KS(52, 74, 408). CD8+ T cell expansion and increased levels of inflammatory cytokines appear to be factors required for KS initiation(157). These indicators of immune function, commonly used to assess HIV/AIDS disease stage, may be useful benchmarks for monitoring and predicting KS disease stage and progression.

Treatment

Though treatment of the underlying immunodeficiency is likely the best and least invasive course of treatment for AIDS-KS(447), other treatment options exist and are routinely used. Local treatments such as cryo, laser and excisional surgery have been used in the past, though primarily for cosmetic treatment and not for systemic management of the disease(25). Chemotherapeutic agents such as doxorubicin and paclitaxel have shown to reduce lesion size(402, 435) though unacceptable toxicity and KS recurrence upon cessation are not uncommon(423).

A number of experimental treatments are still under active study including treatment with thalidomide(445) and retinoids(5, 50). Antiviral therapy has shown the most efficacy for KS treatment. In epidemiology studies and clinical trials, anti-herpesviral agents such as foscarnet and ganciclovir have shown reductions in relative risk of developing KS, though use in treatment of established KS is still controversial(25, 57). In conclusion, as with other types of KS, AIDS-KS is best treated through management of a patient's underlying immunosuppression especially through the use of HAART(95).

1.1.1.4. Iatrogenic Kaposi's Sarcoma

In North America, few studies have been conducted to assess KS incidence in transplant recipients. In Canada, KS was observed in 0.6% of solid organ recipients and 0.1% of renal allograft recipients(126, 388). In the United States, surveillance data reported to the United Network for Organ Sharing (UNOS) show that 0.03% of transplant recipients were diagnosed with KS between 1988 and 2002 (Table 1-2). Of all cases of

post-transplant malignancy reported from 1968 to 1995 to the Cincinnati transplant tumor registry (CTTR), KS comprised 4.1%(338).

Table 1-2. Rates of KS reported to UNOS by transplanted organ

Organ	All Transplants 1988-2002	KS in recipients	Percent of recipients diagnosed with KS 1988-2002
Heart-Lung	799	0	0.00
Heart	32514	14	0.04
Intestine	697	0	0.00
Kidney	169252	46	0.03
Kidney- Pancreas	10574	1	0.01
Liver	56678	11	0.02
Lung	10098	3	0.03
Pancreas	3130	0	0.00
Total	283742	75	0.03

Note: Data provided by the United Network for Organ Sharing (UNOS)(425).

Current estimates of KS incidence in transplant recipients are derived primarily from two sources; retrospective studies which utilize national cancer registry data and retrospective studies which evaluate KS incidence through a process of institutional chart review of patients who have undergone organ transplantation. Both methods of ascertaining KS incidence are likely to underestimate the true rates. However, based upon these studies, in most parts of the world KS is likely to occur in less than one or two percent of all transplant recipients (Table 1-3)(425).

Table 1-3. Reported rates of Kaposi sarcoma among transplant recipients						
Reference	Year	Country and City/Region	No.	%	Total	Population
Shepard et al.(388)	1997	Toronto, Canada	12	0.57	2099	Heart/Lung/Liver/Kidney
Delorme et al.(126)	2003	Québec, Canada	1	0.09	1070	Kidney recipients
Webb et al.(433)	1997	London, England	4	0.31	1304	Kidney recipients
Emond et al.(149)	2002	Paris, France	1	0.67	150	Heart recipients
Francés et al.(165)	2000	Paris, France	9	2.3	400	Kidney recipients
Marcelin et al.(268)	2004	Paris, France	2	1.6	122	Liver recipients
Touraine et al.	1996	Lyon France		0.48	2500	Kidney recipients
Farge et al.(162)	1993	Ile de France		0.45	6229	Kidney recipients
Sheldon et al.(387)	2000	Brussels, Belgium	0	0	200	Kidney recipients
Behrend et al.(31)	1997	Hanover Germany	1	0.07	1497	Kidney recipients
Mitxelena et al.(290)	2003	Bilbao, Spain	6	0.49	1230	Kidney recipients
Gomez et al.(181)	1997	Madrid, Spain	3	0.5	609	Kidney recipients
Anaya et al.(12)	2003	Madrid, Spain	5	0.24	2121	Kidney recipients
Portillo Martin(345)	1992	Santander, Spain	1	0.23	431	Kidney recipients
Zavos et al.(450)	2003	Athens, Greece	18	1.7	1055	Kidney recipients
Montagnino(293)	1996	Milan, Italy	13	1.5	854	Kidney recipients
Busnach et al.(70)	2001	Milan, Italy	2	0.4	499	Kidney recipients
Cardillo et al.(82)	2001	Northern Italy	19	0.84	2261	Heart recipients
Pedotti et al.(331)	2003	Italy	39	1.1	3521	Kidney recipients
Lesnoni La Parola et al.(250)	1997	Rome, Italy	10	3.3	302	Kidney recipients
Cattani et al.(94)	2001	Rome, Italy	7	4.0	175	Kidney recipients
Andreoni et al.(15)	2001	Rome, Italy	4	3.1	130	Kidney and Liver recipients
Sheil et al.(386)	1987	N.Z./Australia	7	0.17	4241	Kidney recipients
Sheil et al.(385)	1997	N.Z./Australia	17	0.21	7909	Kidney recipients
Kim et al.(230)	1998	Seoul, Korea	5	0.3	1600	Kidney recipients
Ecder et al.(146)	1998	Istanbul, Turkey	17	3.1	557	Kidney recipients
Arican et al.(19)	2001	Ankara, Turkey	10	1.0	954	Kidney recipients
Karakayali (221)	1999	Ankara, Turkey	8	0.69	1167	Kidney recipients
Shmueli et al.(389)	1989	Petah Tiqva, Israel	8	2.4	330	Kidney recipients
Qunibi et al.(348)	1993	Riyadh, Saudi Arabia		4.1	630	Kidney recipients
Askari et al.(22)	1999	Karachi, Pakistan	6	0.95	630	Kidney recipients
Al-Mousawi(8)	2001	Kuwait City, Kuwait	9	1.1	800	Kidney recipients
Einollahi (147)	2001	Tehran, Iran	13	0.74	1750	Kidney recipients
Lessan-Peze(251)	2001	Tehran, Iran	18	0.88	2050	Kidney recipients
Margolius (271)	1994	Johannesburg, S.A.		0.5	989	Kidney recipients

Significant morbidity and mortality are associated with a diagnosis of KS among transplant recipients. In the Cincinnati transplant tumor registry, 143 (40.2%) of 356 patients with KS had visceral involvement and 61 (17.1%) of 356 had KS listed as their

cause of death (338). Although the reduction or cessation of immunosuppressive treatment can lead to the complete remission of KS, among patients undergoing remission 65% had graft loss or impaired function, compared to 21% of the overall population of transplant recipients(425). In addition, patients with KS in remission are at increased risk of recurrence(138).

In a recent report of liver transplant recipients in France, 100% of KSHV negative recipients who received a KSHV-infected allograft seroconverted within 6 months post-transplantation. Of those, 50% developed Kaposi's sarcoma and died of multiorgan failure within 1 year post-transplantation (268). Among patients KSHV seropositive prior to transplantation none had developed KSHV related illness at two years of follow-up. This report suggests denovo infection resulting from infected organs may present greater morbidity and mortality than KS acquired from KSHV reactivation in seropositive patients, an observation that appears to also be true for cytomegalovirus(53, 204). It is clear that both primary infection and reactivation with KSHV dramatically increases the risk of malignant and nonmalignant disease(137, 220, 255, 257, 259, 278). However, more studies with larger patient populations need to be done to definitively address the clinical significance of primary infection versus reactivation.

Clearly the most significant risk factor for KS among transplant recipients is infection with KSHV. Though large variation exists in the prevalence of KSHV infection based upon geography, a number of serological studies demonstrate that the seroprevalence of donors and recipients (prior to transplantation) are similar to each other and their general population (Table 1-4)(92, 149, 150, 268, 366).

Table 1-4. Kaposi's sarcoma associated herpesvirus seroprevalence among transplant donors and recipients

Reference	Year	Country and City/Region	Methods	KSHV seropositive pre-transplant	KSHV seropositive post-transplant	KSHV seropositive organ donors	Population
Rosenzweig et al.(366)	1999	Paris, France	Latent IFA (1:150)	29/200 (14.5%)	36/200(18%)		Bone marrow recipients
Francés et al.(165)	2000	Paris, France	Latent IFA (1:100)	32/400 (8%)			Kidney recipients
Challine et al.(99)	2001	Paris, France	IFA to latent, (1:40)		15/99 (15.1%)	8/100 (8%)	Kidney recipients
Milliancourt et al.(288)	2001	Paris, France	Latent IFA (1:100)	0/287 (0%)	6/287 (2.1%)		Kidney recipients
Emond et al.(149)	2002	Paris, France	Latent IFA (1:100)	4/150 (2.7%)	5/150 (3.3%)		Heart recipients
Marcelin et al.(268)	2004	Paris, France	Latent IFA (1:100)	3/122 (2.4%)	7/122 (5.7%)	4/122 (3.3%)	Liver recipients
Deborska et al.(124)	2002	Warsaw, Poland	IgG ELISA	1/88 (1.1%)			Dialysis patients
Sheldon et al.(387)	2000	Brussels, Belgium	Latent IFA (1:50) ORF65/73 ELISA	15/196 (7.6%)	18/199 (9%)	7/210 (3.3%)	Kidney recipients
Regamey et al.(356)	1998	Basel, Switzerland	ORF65.2 ELISA	14/220 (6.4%)	39/220 (18%)		Kidney recipients
Enbom et al.(150)	2000	Sweden	Latent IFA				
			Lytic IFA (1:40)	8/34 (24%)	10/34 (29%)		Bone marrow recipients
			Latent IFA	0/34 (0%)	0/34 (0%)		Kidney recipients
Diociaiuti et al.(135)	2000	Rome, Italy	IFA (1:40)	33/66 (50%)	41/66 (62%)		Kidney recipients
Diociaiuti et al.(135)	2000	Rome, Italy	IFA (1:40)	33/120 (27.5%)			Kidney recipients
Cattani et al.(92)	2001	Rome, Italy	Lytic IFA (1:80)	12/100 (12%)	26/100 (26%)		Kidney recipients
Andreoni et al.(15)	2001	Rome, Italy	Lytic IFA (1:20)	21/130 (16.1%)	34/130 (26%)		97 kidney and 33 liver recipients
Delorme et al.(126)	2003	Québec, Canada	IgG lytic and latent ELISAs		0/150 (0%)		Kidney recipients
Hudnall et al.(203)	1998	Texas, USA	Lytic IFA (1:40)		29/58 (50%)		Kidney recipients
Jenkins et al.(215)	2002	PA, USA	Lytic IFA (1:50)	5/95 (5.3%)	15/95 (15.8%)		Solid-organ recipients
Almuneef et al.(9)	2001	Riyadh, Saudi Arabia	Lytic IFA (1:10) Immunoblot	14/201 (7%)			Patients with end-stage renal disease

Note: Studies assessed post-transplantation KSHV seropositivity at different times; IFA, immunofluorescence assay.

Treatment

Immune reconstitution often leads to remission of KS, therefore reduction or cessation of immunosuppressive therapy is often the first line of treatment for patients with a diagnosis of transplant-associated KS. The dual goal for treatment in this population is KS regression with maintenance of graft function. In a study of twelve patients with post-transplant KS, Duman et al. reported that reduction or discontinuation of immunosuppressive therapy led to complete remission in all patients(143). In the CTTR, remission was achieved in 33% of non-visceral KS cases with complete withdrawal of immunosuppressive therapy alone(334, 335, 337), though the price of remission of post-transplant associated KS was high (Figure 1-3). Similarly, Moray et al. found among renal transplant recipients with KS that 4/7 (57%) patients with lesions confined to the skin showed complete regression of all lesions after reduction of immunosuppression(303).

Early diagnosis of KS appears critical, as cases with non-visceral involvement can often be managed with the reduction of immunosuppressive therapy. However, patients with systemic disease do not respond favorably to adjustments in the therapeutic regimens alone and require second-line treatment which can include surgical excision, radiotherapy, cryosurgery, and chemotherapy. Prognosis of patients with systemic KS is often poor and marked with high rates of graft loss and mortality.

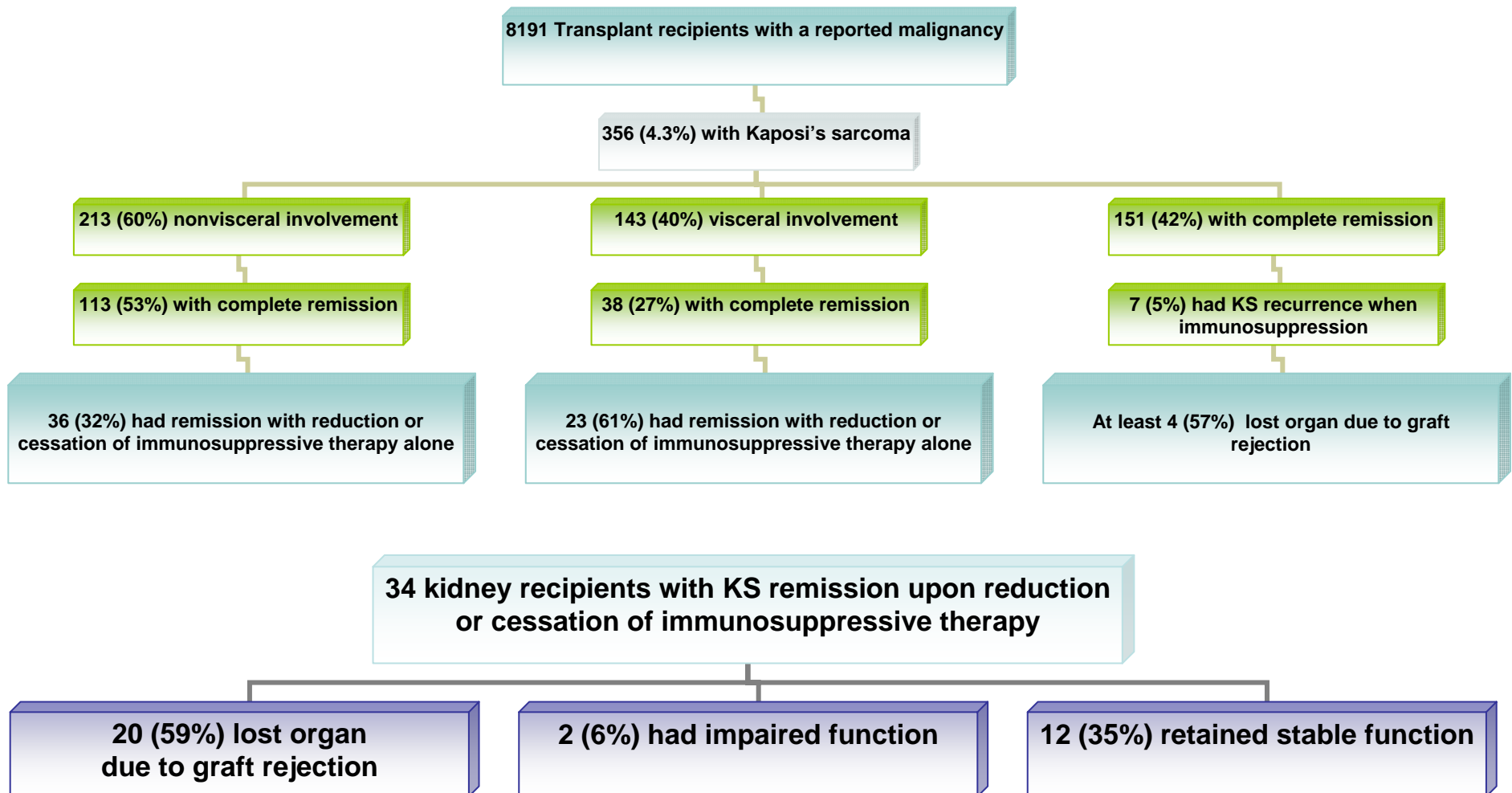


Figure 1-3. Cincinnati Transplant Tumor Registry Data 1968-1995

Though calcineurin inhibitors have increased overall graft and patient survival rates compared to treatment with azathioprine and glucocorticoids alone(186), KS is more common among patients treated with cyclosporine compared to azathioprine(7, 336). In a recent case report, conversion from cyclosporine treatment to a new immunosuppressive agent, sirolimus, led to complete regression of KS lesions among two renal transplant recipients with KS(75). Though studies have not yet assessed the risk of KS development on a regimen of sirolimus compared to other immunosuppressive therapies, these reports suggest that sirolimus instead of calcineurine inhibitors may maintain graft viability while simultaneously protecting against initial KS development.

1.1.2. Primary Effusion Lymphoma

Primary effusion lymphoma (PEL), also known as body cavity based lymphoma (BCBL), is a rare subset of AIDS-related non-Hodgkin's lymphoma, which is a serous effusion isolated primarily in the pleura, pericardium or abdominal cavities. In rare circumstances the PEL may present as a solid tumor mass(119). It is estimated that PELs account for about 3% of AIDS-related lymphomas(80). In 1995, Cesarman et al. demonstrated that KSHV was present in all AIDS-related PELs(98), and subsequently others have confirmed these findings(79-81, 176, 222, 223, 321, 327). Of epidemiologic and clinical importance, Cesarman reported that approximately 25% of AIDS patients with PEL have KS comorbidity.

Though KSHV is nearly always identified in patients with PEL, four case reports of PEL, among patients with chronic hepatitis C-related (HCV) cirrhosis of the liver, have failed to identify KSHV among these patients. All four patients have been described as KSHV negative, HIV negative and EBV negative calling into question whether HCV or other unidentified virus may also be responsible for a small subset of PELs(21, 189, 207, 323).

Though these four case reports exist, the broader scientific thinking remains that KSHV is associated with all forms of PEL.

PEL is most common among immunocompromised patients(216, 247, 306), however, in rare circumstances, PEL has been observed in otherwise immunocompetent individuals(346, 409). The epidemiology of PEL is similar to that of KS, in that age of presentation of disease is earlier in HIV-infected individuals (42 years) compared to non-HIV infected individuals (73 years), and most cases are among HIV infected men who have sex with men, though intravenous drug use has been associated with PEL in some European studies(233, 234). Overall prognosis of patients with PEL is poor with mortality often observed within 3 months of diagnosis(17, 98, 390). Because of the rarity of PEL, much is still to be elucidated regarding causal cofactors among immunocompetent patients. However, sufficient evidence exists to support KSHV as a causal cofactor in the development of PEL, based largely upon the strength of association.

1.1.3. Multicentric Castleman's Disease

Castleman's disease was first described 50 years ago by pathologist Dr. Benjamin Castleman of Massachusetts General Hospital in a series of case reports(86-88). Since that time, the unique pathological characteristics of the disease have been observed and multiple variants of the disease have been described. One form of Castleman's disease, a lymphoproliferative disorder highly associated with KS, is Multicentric Castleman's Disease (MCD). The diagnostic criteria for MCD are less well defined than other forms of Castleman's disease with patients often presenting with systemic symptoms including; fever, weight loss, splenomegaly, and hepatomegaly. In addition, patients with MCD have a high rate of KS as a comorbidity(84). Up to 15% of MCD patients may exhibit the rare POEMS syndrome

(polyneuropathy, organomegaly, endocrinopathy, multiple myeloma, skin changes)(342).

Prognosis in patients with MCD is poor with sepsis, multi-organ failure or lymphoma the most common causes of death(194, 195).

Due to the rarity of the disease, the pathoetiology and epidemiology of MCD are not fully understood. As one example, the population prevalence rate of MCD is unknown. A large retrospective study from a cancer center estimated in 1996 the total number of cases in the United States to be from 30,000 to 100,000(84). This may be an underestimate however because it is assumed that AIDS-associated MCD is likely under diagnosed(192) due to comorbidities which may be preferentially diagnosed among this patient population.

Early in the discovery of MCD a viral etiology was suspected. Based upon the pathophysiology of the virus EBV was a suspected causal agent. A number of attempts to identify EBV in MCD patients were undertaken and identification of the virus was inconsistent(113, 188, 305, 315). Based upon these results, and because of the higher prevalence of EBV in the general population, EBV is thought not to cause MCD.

MCD can be divided into two separate entities, a plasma cell variant type and a hyaline vascular type. The plasma cell variant is highly associated with KSHV and the hyaline vascular type rarely associated with KSHV. Though EBV is likely not a factor in MCD, several lines of evidence exist which demonstrate that KSHV does play a role in the plasma cell variant of MCD. First, studies of KSHV prevalence among patients with MCD is extraordinarily high with prevalence rates among HIV positive individuals reported at 100%(399, 405). Among non-HIV infected patients with MCD, only small studies have been conducted but the rate of KSHV in these studies has been reported at 0, 41, 81 and 100 percent(229, 399, 405, 446, 454). It is assumed that the increase in prevalence among MCD

patients with HIV infection compared to the uninfected is explained by the increased rate of KSHV naturally observed among HIV infected individuals(432), though this has not been proven. In addition, the low rate of KSHV observed in non-HIV infected individuals is an association observed with the hyaline vascular MCD form.

Additional evidence that KSHV is a factor in MCD is the observation that KSHV DNA viral load in PBMCs and plasma increases in association with symptom flare-ups of MCD in HIV positive patients. The counter-trend has been observed as well, where it has been noted that symptoms of MCD regress when KSHV DNA load decreases or becomes undetectable by PCR(37, 51, 58, 85, 111, 182, 313). Lastly, as described by Casper et al., antiviral medications, particularly agents used to treat herpesviral infections, have been observed to lead to regression of symptoms in HIV-MCD patients(84, 85).

It is clear that KSHV plays a significant role in the pathogenesis of the plasma cell variant of MCD and patients in whom KSHV infection has been established are more likely to have poorer outcomes. The reason for this is likely because the systemic pathologies observed in MCD are the result of over expression of the cytokine interleukin 6 (IL-6). Elevated serum levels of IL-6 are a hallmark of MCD. KSHV expresses a viral analogue of IL-6, viral IL-6(vIL-6), which shows high sequence homology to human IL-6 (hIL-6)(297), and symptoms including pancytopenia, thrombocytopenia and shock can be induced by high levels of vIL-6 in MCD patients(432). The KSHV cytokine vIL-6 contributes to the systemic pathologies observed among patients with MCD and some therapies which target KSHV have been shown to be effective in treatment.

Taken together the evidence supports a strong association between KSHV and the plasma cell variant of MCD. This association is thought to be causal, though due to the rarity

of the disease, and the fact that most of the current knowledge is based upon case-reports and case-series, establishing temporal associations is a difficult task. The next step in elucidating epidemiological questions regarding KSHV and MCD will likely require case-control studies with well reasoned controls to obtain a better understanding of the role KSHV plays in the natural history and pathogenesis of this disease. In addition, a shift in nomenclature or a concentrated effort to clearly describe MCD as two separate entities (plasma cell versus hyaline vascular) would aid in further clarifying the role the virus plays in MCD.

1.1.4. Other Disease Associations

In a report of three patients, Luppi et al. provide evidence that KSHV infection is likely responsible for post-transplant associated bone marrow failure(258). In a similar report of one patient, Cuzzola et al. report a similar finding where KSHV was responsible for bone marrow failure after transplantation in a 17 year old girl(121). Though larger studies are required to make firm conclusions and elucidate pathological mechanisms, these case reports are highly suggestive that this association is accurate and may be of important public health significance.

Apart from the diseases described above (KS, MCD, PEL and perhaps post-transplant associated bone marrow failure) there is not sufficient evidence to support a consistent exposure-disease association between KSHV and any other clinical condition. However many associations have been reported later to be refuted entirely or have yet to be confirmed in a robust manner. Likely the most controversial of these is the reported association between multiple myeloma (MM) and KSHV.

Shortly after KSHV was first identified, a number of studies were conducted to assess viral prevalence among clinical populations with various malignancies and conditions of unknown etiologies. On the whole, these studies were largely null and included studies of MM

where the virus was unable to be identified(98, 176, 321, 327). A subsequent report found that KSHV DNA was present in bone marrow dendritic cells of MM patients(360). Based upon these findings a number of groups performed studies of KSHV and MM with varying results. Some confirmed the findings that KSHV DNA was present in MM biopsies(6, 32, 66, 67) while many others were unable to identify the virus by PCR in bone marrow biopsies or peripheral blood(60, 90, 112, 277, 289, 325, 341, 411-413, 420, 448). As a result multiple serological studies were conducted and only one(172), using an immunoblot technique, found evidence that KSHV played a role in MM(2, 60, 90, 261, 266, 277, 289, 325, 340, 341, 370, 436).

The current evidence appears not to support a KSHV/MM association. Recently, the largest prospective cohort study conducted to date (1,133,000 individuals) was sampled in an attempt to be the definitive word on KSHV and MM. The Joint Nordic prospective study group found that KSHV is not associated with MM in a convincing manner(418).

Isolated case reports of patients with sarcoidosis and KS led investigators to look for KSHV DNA among this patient population(116, 128). A positive association between KSHV and sarcoidosis was reported (129, 130) but later refuted by a number of different laboratories(34, 166, 169, 232, 244, 262, 406). In a similar manner KSHV has been associated with pemphigus(214, 284, 285, 396), though the strength of this association has been questioned(40, 91, 107, 145). A number of other associations, including primary and secondary pulmonary hypertension have been found but ultimately refuted (see chapter 3). For a more comprehensive discussion of the diseases in which KSHV does not appear to be associated, Cohen et al. have recently put together a compendium of the populations in which studies have been conducted on KSHV prevalence (reviewed in (106)).

1.2. Etiology of Kaposi's Sarcoma

Substantial evidence exists to support the argument that KSHV is the etiologic agent of KS, though in most individuals infection alone will not lead to disease. In AIDS and transplant-associated KS, underlying immunosuppression appears to be an important and necessary cofactor. Though perhaps not sufficient, KSHV is necessary for KS. The following sections are designed to demonstrate in a systematic way that KSHV is the causative agent of KS. Using Sir Austin Bradford Hill's criteria for causality as a model, the causal association of KSHV with KS is explored.

Bradford-Hill Criteria for Causality

In an attempt to demonstrate the difficulty in characterizing the differences between simple associations and causal associations, Hill described in 1965 a set of criteria which could aid scientists and epidemiologists in systematically compiling evidence to determine if among an observed or hypothesized exposure-disease relationship the exposure of interest actually causes the disease(61, 196). Hill's original criteria include strength of association, consistency, specificity, temporality, biological gradient, biological plausibility, coherence, experimental evidence and analogy. He notes that some of the criteria are of more importance than others in determining causality and not all criteria can or need to be demonstrated to establish evidence for a causal association. However, as an exercise in assessing whether KSHV is the causative agent of KS the criteria are used as a model to evaluate this exposure-disease relationship.

1.2.1. Strength of Association

The first criterion Hill describes is strength of association. Specifically in this setting, we must address the following: is the prevalence of KSHV among patients with KS great enough to reasonably support an actual and important association? If an observed association is

particularly strong it provides evidence that the observation is less likely to be comprised entirely of confounding factors or information bias. In early investigations of smoking and lung cancer it was observed that among individuals who smoke, mortality from lung cancer was about 10 times greater than among non-smokers. Based upon this information alone, many believed that smoking was likely the cause of the excess cases of lung cancer. As a comparison, for KSHV, the odds of detecting the virus in KS lesions compared to a normal tissue site exceed 100(317, 373).

KSHV DNA is routinely detected in greater than 95% of all KS lesions by PCR techniques. In studies using unaffected skin areas from the same patients with KS, the chance of detecting KSHV DNA is greatly reduced. Additionally, among control tissues obtained from patients with no clinical signs of KS, KSHV DNA is rarely amplified in PCR reactions(10, 69, 114, 144, 170, 246, 269, 298). These studies are highly suggestive that KS lesions harbor KSHV at a rate much higher than control tissues.

In addition to tissue studies, which can identify viral DNA at the site of KS lesions, serological evidence supports an association between KSHV and KS. Antibodies to KSHV are more common among patients with KS compared to patients without KS. Additionally among patients known to be infected with KSHV, antibody titers are higher among patients with KS compared to non-KS groups(41, 76, 103, 241, 392). Seroepidemiology studies have characterized worldwide seroprevalence rates of KSHV. KS disease rates in endemic regions roughly follow the same geographical pattern. Though using trend data and ecological associations are not criteria in which to base definitive “proof” of causality, the strength and consistency of these observations are difficult to dismiss outright.

Overall the strength of association between KSHV and KS is strong with ample evidence to suggest that KSHV is consistently present in KS tumors and antibodies to KSHV are common among patients with KS, though the virus is not ubiquitous in the general population.

1.2.2. Consistency

Hill defines this criterion the following way; has the observed association been repeatedly observed in different places, circumstances and times? For the KSHV-KS disease exposure association the evidence is overwhelming that indeed the consistency criterion is fulfilled. In studies using tissue from KS lesions, different laboratories using different techniques are highly consistent in their ability to detect KSHV in all forms of KS. In the past, variation did exist in detection rates based upon PCR detection methods and the quality and type of tissue examined. For example, it has been noted that DNA detection rates are higher among fresh lesions compared to stored paraffin embedded tissues(100, 101, 298, 300). Though these problems have been noted, multiple studies conducted in more recent years show a high rate of concordance in the ability to detect KSHV DNA in KS lesions. Scientific consensus has been reached with respect to the consistency of these findings.

1.2.3. Specificity

On specificity, Hill states “If specificity exists we may be able to draw conclusions without hesitation; if it is not apparent, we are not thereby necessarily left sitting on the fence.” In general, when discussing viral etiologies, strict specificity is not a criterion in which one is able to draw such causal conclusions without hesitation. Because KSHV is clearly associated with pathologies other than KS (as discussed in section 1.1.), adherence to the specificity criterion in the classical sense would appear not to be met. However a number of viruses,

including other herpesviruses, are known to produce multiple syndromes, diseases and disorders. Specificity of association may be useful in assessing causality of environmental exposures, though in assessing the pathoetiologies of potential tumor viruses it may not be as important a criterion.

In a sentinel work addressing this issue, Moore and Chang in the American Journal of Epidemiology, lay out a new way of thinking with respect to specificity of effect using as an example the closely related Epstein-Barr virus (EBV)(299). Because specificity of effect could not be demonstrated for the EBV-Burkitt's lymphoma association, some factions continued to argue that EBV was not causal for the disease even when faced with molecular proof. Moore and Chang argue that though Hill's criteria are of great utility, molecular techniques which have the ability to often elucidate causal associations alone should not continue to be left ignored by those who are disciples of the 1965 criteria.

It is not that KSHV infection is not specific to KS. To the contrary, as described in the previous "strength of association" section, KSHV DNA is localized to the site of KS lesions. However, KSHV is not uniquely specific to KS, as PEL and MCD are also clearly caused by the virus. In regard to the KSHV-KS interaction we cannot "draw conclusions without hesitation" with the specificity of effect criterion. However, it is likely that few tumor causing viruses show this type of specificity.

1.2.4. Temporality

For an exposure to be truly causal the correct temporal relationship between exposure and disease must be established. KSHV must precede and predict the development of KS to be considered the etiologic agent. The type of study design required for assessing the proper sequence requires a cohort of individuals who are initially disease free and exposure free. The

cohort then needs to be followed over time to assess the actual sequence of events. Specifically, does exposure (KSHV infection) always precede disease (KS)? The 1996 *AIDS* paper “KSHV infection prior to onset of KS” seemed to provide sufficient evidence that the correct temporal trend was observed(302). This study tested serial PBMC samples from HIV infected individuals prior to and after the development of KS. Patients who developed KS were at substantially greater risk of KSHV infection prior to development of disease compared to patients who did not develop KS. In another population of HIV infected individuals, patients who had detectable levels of KSHV DNA present at enrollment were at substantially greater risk of developing KS compared to the participants who were not PCR positive(438). Since those early studies, additional evidence has emerged in numerous studies of different designs and methods that confirm these findings (Table 1-5).

Table 1-5. Temporal association of KSHV and KS: evidence which supports the condition that exposure precedes disease

Evidence	Selected Reference List
DNA detection and proliferation prior to KS in HIV cohorts	Moore et al. 1996(302) Whitby et al. 1995(437)
KSHV seroconversion prior to KS in HIV cohorts	Gao et al. 1996(173) Melbye et al. 1998 (283) Renwick et al. 1998(359)
Serological detection of KSHV prior to organ transplantation and KS	Andreoni et al. 2001(15) Jenkins et al.2002(215) Luppi et al. 2000(256) Parravicini et al. 1997(326)
Primary infection after transplantation leads to KS	Collart et al. 2004(108) Luppi et al. 2000(257) Luppi et al.2003(254) Regamey et al. 1998(356)

Note: References presented reflect a selection of important works and are not a complete list of all studies performed on KSHV/KS temporality.

More recent studies done in the transplant setting have elegantly shown the temporality of the KSHV-KS relationship. Patients negative for KSHV can be infected through solid organ transplantation and subsequently develop KS(30, 296). Marcelin et al. showed that among liver transplant recipients, 100% of KSHV negative recipients who received a KSHV-infected allograft seroconverted within 6 months post-transplantation. Of those, 50% developed Kaposi's sarcoma and died of multiorgan failure within 1 year post-transplantation (268). This study nicely demonstrated the temporality of infection and disease. Evidence has shown that in all cases in which the temporal association between KSHV and KS has been assessed that KSHV precedes KS. The Hill criterion of temporality appears to be met in the present setting.

1.2.5. Biological Gradient

Traditionally the biological gradient criterion has been thought of in terms of a dose-response relationship. As an example, the risk of lung cancer is not simply a dichotomous association. It has been shown that as the number of cigarettes consumed increases so too does the associated risk of disease. In terms of dose response the question would be; is more exposure to KSHV associated with KS? To know for certain the amount of exposure to KSHV an individual encounters is not possible. However, in one study, KSHV superinfection, as evidenced by identification of multiple strains of KSHV in the same individual, has been shown among patients with and without KS(39). In most viral diseases superinfection is a risk factor for disease development, though in the setting of KSHV the actual significance of superinfection is currently unknown. For most viral infections, defining dose response in this manner may not provide the most utility in attempting to understand exposure-disease associations.

In a similar consideration of KSHV using the Hill criteria, Sarid et al. define the biologic gradient criterion in terms of the location of KSHV DNA within individuals with KS. Most studies have demonstrated that detection rates and KSHV DNA viral load is greater in biopsies taken directly from a KS lesion compared to unaffected tissue sampled from the same patient(10, 55, 100, 144, 243, 246, 269, 298, 311). Exceptions exist among KS patients with advanced KS and disseminated infection(69, 114). However, based on this definition of the biologic gradient, KSHV DNA is present more frequently in KS lesions compared to unaffected tissues and therefore fulfills the criterion.

1.2.6. Biological Plausibility

As with all members of family *Herpesviridae* characterized to date, KSHV is a large virus and has a linear double stranded DNA conformation. A member of the gammaherpesvirus subfamily, KSHV is most closely related to herpesvirus saimiri of squirrel monkeys and Epstein-Barr virus (EBV) of humans(295). Figure 1-4 represents the phylogentic relationship among KSHV and other herpesviruses. Gammaherpesviruses are known to infect lymphocytes and cause tumors in both animals and humans. For example, EBV is known to cause Burkitt's lymphoma and nasopharyngeal carcinoma(322, 419, 449). Because related viruses have been shown to be oncogenic it is certainly plausible that KSHV causes KS.

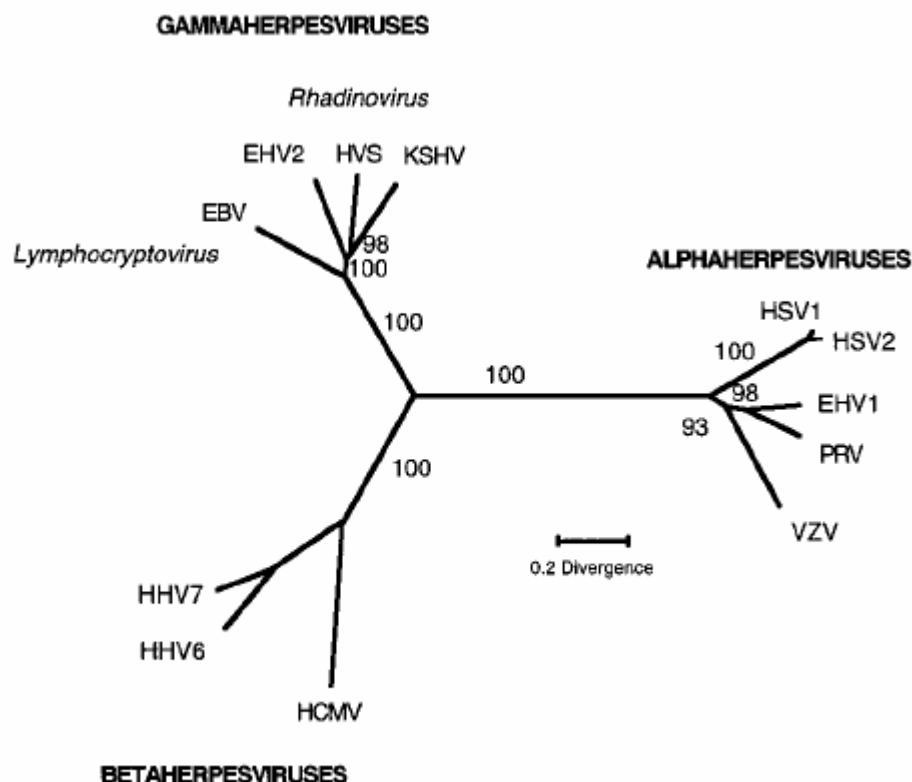


Figure 1-4. Phylogenetic tree of KSHV and other herpesviruses. Figure from Moore 1996 et al. J. Virol70:549-58.(301)

In Hill's original description of the criterion, he minimizes the importance of plausibility. He argues that biologic plausibility is dependent upon the biological knowledge of the day. Therefore, a concept which is seemingly implausible may at some point become plausible given novel scientific findings uncover mechanisms which up to that time had gone unobserved. This is precisely what has happened in the setting of KSHV and KS. In the 1980's, for many, the thought that KS was caused by an infection other than HIV was entirely implausible. For a minority, (35) it seemed patently clear that KS was caused by an infectious agent distinct from HIV. Based upon new scientific findings(100, 298), namely the

identification of KSHV and research establishing that KSHV encodes multiple viral oncogenes, mass opinion shifted and now most scientist agree that not only is it biologically plausible but scientifically proven that KSHV is the etiologic agent of KS.

1.2.7. Coherence

Coherence is not unlike biological plausibility but interpretation is made using only the biological understanding that is currently known of the exposure and disease relationship. The cause and effect interpretation of the available data should not seriously conflict with the generally known facts of the natural history and biology of KS. When Beral et al. proposed that KS may have an infectious disease etiology certain assumptions were made that proved later to be correct. For example, KSHV is not a ubiquitous infection but rather has a worldwide prevalence that is quite low. KSHV is largely asymptomatic among those infected but among groups at high risk for KS, especially among individuals who are immunocompromised, KS can be quite aggressive. Infection with KSHV appears to be highly associated within individuals already at high risk for KS, especially among men with AIDS(227, 265, 275, 287, 326, 397). In addition, the geographic distribution of KSHV infection (Figure 1-7) appears to be highly associated with the global prevalence of KS disease. In conclusion, there appears to be no serious conflict or contraindication, with what is known about the biology and natural history of KS, in the assumption that KSHV causes KS.

1.2.8. Experimental Evidence

Though perhaps the most convincing way to demonstrate causality is through direct scientific experimentation, in the setting of KSHV and KS, human experimentation is clearly unethical. Therefore a strict interpretation of the criterion would yield it unfulfilled. However, indirect experimental evidence through clinical trials can provide useful information when

attempting to establish causality. Multiple studies establishing the efficacy of the anti-herpes drugs ganciclovir and foscarnet have demonstrated that these agents prevent the onset of KS(117, 178, 217, 242, 272, 291, 351, 364). In one clinical trial where patients were randomly assigned treatments, Martin et al. show that a course of oral ganciclovir reduced the risk of developing KS by 75%. More dramatically they showed that intravenous treatment reduced the incidence by 95%(272). These results are highly suggestive that treatments with agents used to target herpes infections are protective against KS onset. Though this is not direct evidence the strength of these observations bolsters the argument that KSHV causes KS.

1.2.9 Discussion of KS and Causality Criteria

In creating these criteria for causality, Hill makes clear that it is not possible to lay down hard and fast rules by which causality can be definitively assessed. However these nine principles do provide a framework in which we can begin to make informed decisions regarding causal associations. In assessing whether KSHV is the etiologic agent of KS the weight of evidence using the Hill criteria is strongly in favor of the hypothesis. Factors in addition to KSHV, namely immunosuppression, may also be required for disease however KSHV appears to be a necessary condition in all forms of KS.

1.3. Methods for Detecting KSHV infection

A number of techniques have been employed for defining infection in individuals and include both molecular and serological methods. KSHV has been identified in cells, tissues, and body fluids through exploiting these different approaches individually and in combined formats.

1.3.1. Detection of KSHV by Polymerase Chain Reaction (PCR)

PCR methods are most often used for the detection of KSHV DNA. Initial studies have demonstrated that at least 95% of KS lesions harbor KSHV DNA that can be amplified by PCR(98, 100, 200, 211, 373, 378, 379). However, detection of KSHV DNA load in peripheral blood samples from patients with KS is substantially lower and is often not detected, though nested PCR has been shown to increase the ability to detect DNA (76, 302, 391, 400, 438). PCR detection during latent infection among patients without active KS is likely low and therefore the utility of PCR as a tool for definitive diagnosis in the general population is limited. In addition, PCR contamination has been observed as a potential problem in testing samples for KSHV(227, 434) and has led to a number of disease associations(36, 129, 352, 360) which were later proved to be false due to contamination(2, 97, 172, 244, 341, 354). Because of these disadvantages and controversies, PCR has proven not to be a reliable method for large scale population screening studies of KSHV.

1.3.2. Detection of KSHV by Immunohistochemistry (IHC)

KSHV can be detected in paraffin-embedded human tissue using IHC and is used as a technique for pathological confirmation of KS. A variety of antibodies to KSHV antigens are available for IHC, though the reliability of some of these commercially available antibodies has been questioned. This technique has proven useful for detecting KSHV among patients

with Castleman's disease, among tumors from patients with primary effusion lymphoma (PEL) and from lesions derived from KS patients. Because tissue is required and antibody reactivity varies this technique is considered useful only for pathological diagnosis and is not generally available for screening studies. Some studies have used IHC to determine KSHV prevalence in tissues taken at autopsy from patients with no serological evidence of KSHV infection(110). In a previous report we have pointed out that IHC may be prone to misdiagnosis of uninfected individuals due to the inherent subjectivity of the method and also the potential for nonspecific binding of antibodies(240).

1.3.3. Detection of KSHV by Serology

It is likely that the most efficient and accurate method for diagnosis of KSHV infection is through serological techniques. Katano and colleagues identified a number of antigens that are useful for examining KSHV in sera from KS patients. These antigens are encoded by open reading frames (ORF) 6, 8, 9 25, 26, 39, 59, 65, 68, 73, K8.1A and K8.1B(226). Various serological methods have been developed for KSHV detection. Immunofluorescence assays which target both latent and lytic antibodies, immunoblot assays, and enzyme-linked immunosorbent assays, which have primarily been used to target lytic antibodies, have all been used with varying levels of sensitivity and specificity.

Serological detection of KSHV by Western blot

Western blotting for KSHV has been performed targeting a variety of viral proteins(173, 287, 349, 353, 391). Gao et al. first showed that sera from patients with KS were reactive to the latent protein encoded by ORF73 (LANA) by Western blot with 80% sensitivity. Figure 1-5 is an example of a typical Western blot. This protein, which is localized to the nucleus has been the primary latent antigen used in the serological assays of

other formats which followed. Though LANA and other viral antigens have been run on Western blots, for serological testing, this method has been largely replaced by IFA and ELISA assays due to the technical aspects associated with this technique and the relatively low specificity of the assay.

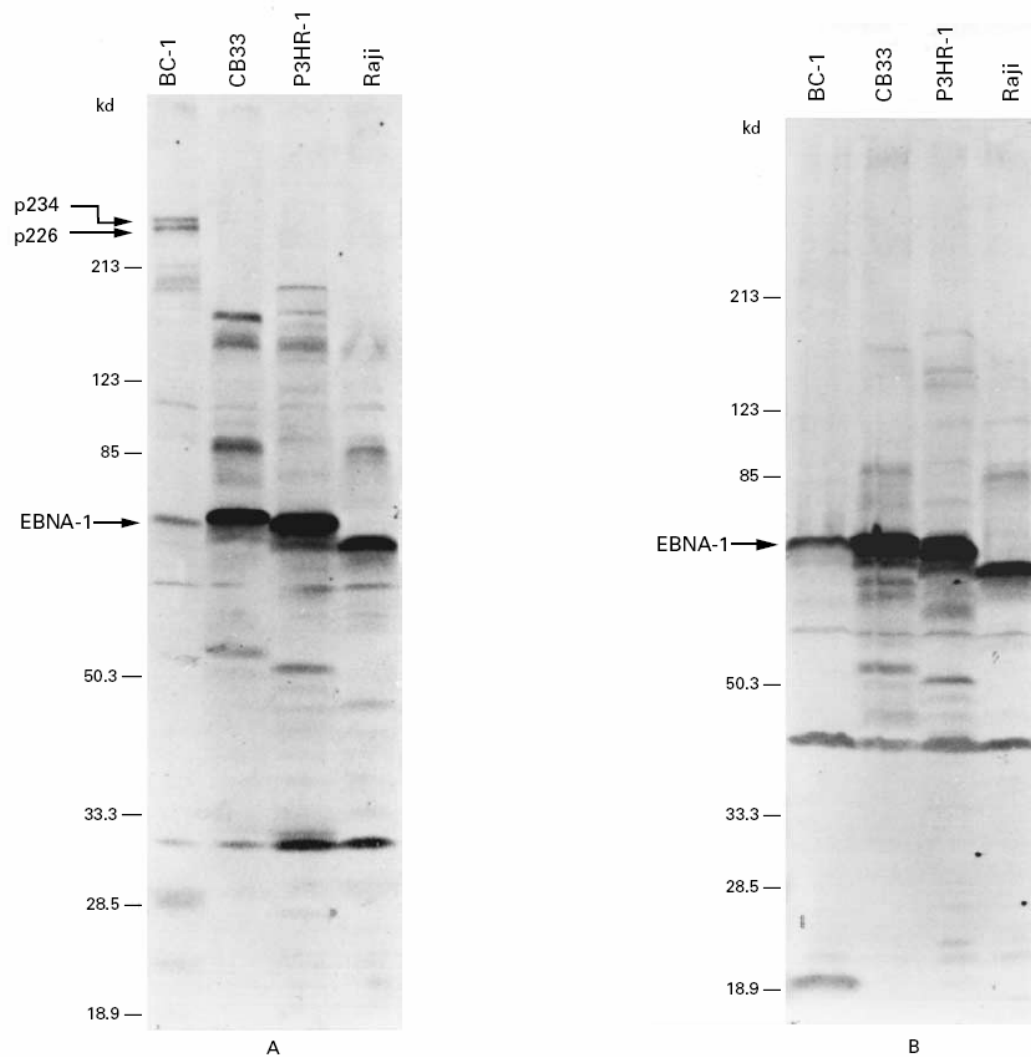


Figure 1-5.
Representative Immunoblots of Serum Samples from a Patient with AIDS-Associated Kaposi's Sarcoma (Panel A) and a Patient with AIDS but No Kaposi's Sarcoma (Panel B). Reproduced from Gao et al. 1996 N Engl J Med. 335:233-241.(173)

Serological detection of KSHV by Immunofluorescence Assays (IFA)

One of the first serological tests developed for detection of KSHV in sera was the indirect IFA which targets LANA. An example of an IFA from an AIDS-KS patient is presented in figure 1-6. LANA is composed primarily of the gene product ORF73 and is responsible for the episomal maintenance of HHV-8 genomes in latently infected cells. The three most commonly used cell lines used to prepare the test are BC-1, BCP-1 and BCBL-1(174, 249, 287, 301, 357) and are all chronically infected with KSHV and continuously express latent antigens. The BC-1 cell line is coinfectd with Epstein-Barr virus (EBV), a herpesvirus related to KSHV, and cross-reactivity between the viruses has been observed(13, 287, 399). The BCBL-1 cell line is EBV negative which has allowed for the creation of more sensitive IFAs for the detection of KSHV(357).

Sera From Patient With KS

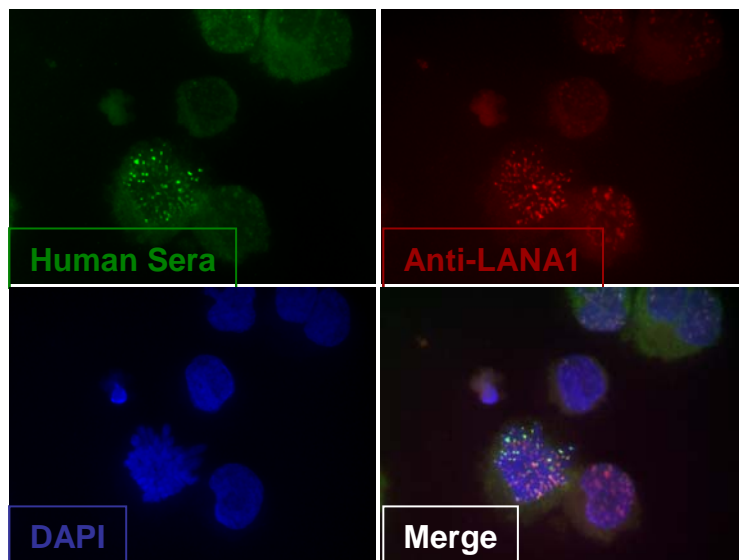


Figure 1-6. Immunoflourescence assay staining for an individual infected with KSHV

One method, which has been used for increasing whole cell IFA sensitivity, is induction of lytic antibody production in the cells through treatment with phorbol esters (TPA treatment) or butyrate(71, 103, 249, 397). This method allows for detection of structural (lytic) proteins through immunofluorescence. However, evidence suggests that due to the homology of structural proteins across herpesviruses this may be a mechanism for cross-reactivity. For example, Moore et al. demonstrated that some structural proteins of KSHV show high homology, up to 50%, with EBV(301). Therefore patients identified as KSHV positive by this method may instead be infected with the more common EBV virus.

In one study of US blood donors, the KSHV seroprevalence rates were significantly higher using the lytic IFA compared to other serological techniques(333). In published studies where assay performance was directly compared between the two methods, the lytic IFA consistently produced more positive results compared to the latent IFA (Table 1-6).

Table 1-6. Direct comparison of KSHV seroprevalence using lytic and latent IFAs as previously reported

Population	Prevalence Lytic IFA	Prevalence Latent IFA	Percent Difference	Reference
Outpatient hospital attendees – Cameroon	50.5	25.3	50.0%	Serraino et al. (2002)(384)
Pregnant women – Uganda	44.8	27.0	39.7%	
Children – Egypt	42.4	6.4	84.9%	
Italian blood donors	18.7	12.7	32.1%	
KS patients	88.0	88.0	0.0%	Enbom et al. (2000)(150)
Swedish blood donors	20.0	0.6	97.0%	
Individuals with high risk sexual behavior	30.0	3.5	88.3%	
Bone marrow transplant recipients – pre-transplant	24.0	0.0	100%	
Bone marrow transplant recipients – post-transplant	29.0	0.0	100%	Hudnall et al. (2003)(202)
US blood donors	23.0	5.0	78.3%	
KS patients	93.0	85.0	8.6%	
Italian blood donors	22.0	2.6	88.2%	
KS patients	83.3	66.6	20.0%	Rezza et al. (1998)(362)
STD patients	48.8	13.7	71.9%	
Italian blood donors	27.7	2.1	92.4%	

In addition, assay comparison studies show a high rate of discordance between the lytic IFA compared to other serological techniques; techniques that have a high degree of inter-assay agreement. This supports the argument that though sensitive, the lytic IFA is likely of little value in regard to specificity. An additional problem, which has been noted with the IFA, is the occurrence of nonspecific reactivity among sera with high lipid content and among

certain patient populations. Populations which have been shown to produce this nonspecific reactivity include sera from individuals with a high number of sexual partners, patients with parasitic infections and among people exposed to a high number of pathogens(102). In addition, patients with autoimmune disease or other hematological perturbations may be more likely to cause false-positive KSHV test results (chapter 2).

Overall the IFA assays have provided initial estimates of KSHV infection rates in various populations, have provided evidence that KSHV is the causative agent of KS and are easier and more quickly performed compared to immunoblotting. However, as an independent diagnostic tool, the latent IFAs lack a certain degree of sensitivity (~80%) and the lytic IFAs lack specificity (<80%).

Serological detection of KSHV by Enzyme-Linked Immunosorbent Assays (ELISA)

As with the other serological techniques for defining KSHV infection, ELISA systems are used only in laboratory research to diagnose infection because no single ELISA assay has demonstrated consistent sensitivity and specificity high enough to be of clinical utility. A few ELISA formats are available commercially; however, the majorities exist as home-made preparations. This has made interpretation of seroepidemiology studies difficult because the techniques are often unique to an individual laboratory and specific and complete methodologies are often left unexplained. In addition, as with the IFA assays, performance of the ELISA assays appears to be highly dependent upon the study population being tested(3, 102, 372).

Various antigen preparations have been used for the ELISA serological methods and include the use of whole viral lysates, recombinant peptides and synthetic peptide preparations. Most KSHV ELISA assays are based upon detection of lytic antibody to KSHV. Many use

specific antigens, such as ORF65, which has been developed to target antibodies to the KSHV major capsid protein(329, 350, 391). One of the other more commonly used lytic ELISA assays is based on ORF K8.1 (238) which has been consistently shown to have higher sensitivity and specificity than many of the other serological techniques. This is likely due to the fact that K8.1 is highly immunogenic and has no homolog in other herpesviruses(208, 209, 238, 252, 400).

ELISA assays which target lytic viral antigens have been found to be of highest sensitivity and overall accuracy in assay comparison studies, though concordance between these assays has varied. Rabkin et al. performed an assay comparison study which found considerable variation between the assays(350). In a similar study, Enbom et al. noted similar inconsistencies in sensitivity and specificity with questionable consistency between different methods(150).

Using second-generation KSHV serological assays, Schatz et al. found higher concordance rates(375). However, though different laboratories participated in the study, the assays used in the study largely targeted the same viral antigens. No latent class analysis or other type of weighting was performed to account for the correlation between assays. Therefore the higher inter-assay concordance reported may be in part to inherently low within-assay variation as opposed to concordance between completely different serological assays. If this is indeed the case, it does provide evidence that the overall reproducibility of the methods are high (between-laboratory variation using similar methods is low).

A comparative assay study conducted more recently among US blood donors by Pellett et al. took into account within-assay similarities. Using a latent-class analysis they determined the rate of KSHV infection among US blood donors to be 3.3%(333). The laboratories which

participated in the study used multiple assays to define KSHV seroprevalence and the outcome measure of this study was a laboratory's definition of KSHV positivity; therefore, individual assay performance was not directly reported. However, of the 40 blinded positive controls (from highly seroreactive KS patients) all laboratories identified the specimens accurately as KSHV positive. Among the 1000 blood donors that were tested the range of seropositivity was 0.5% (5/1000) to 3.7% (37/1000). All of the laboratories found low rates of KSHV positivity in the group of blood donors, however, the kappa values reported were very low. This suggests that if the same study was conducted in a population where the KSHV seroprevalence rate was higher (nearer 50%) assay discordance would be very high and laboratory agreement would be low.

Attempts to design an ELISA assay which targets LANA have been reported with varying levels of success. Initially, Olsen et al. mapped ORF73 (LANA1) for peptides which could potentially be used in an ELISA format(318). Though 13 potential peptides were identified, individually, sensitivities of the products were low (11%-44%). In a combined format the sensitivity of the assay was increased to 78%. Using the Semliki Forest virus system, Corchero et al. produced KSHV LANA for use in an ELISA format(115). They report however that lytic antigens are universally more useful than latent antigen-based ELISAs in detecting KSHV infection. The methods described by these two groups have not been adopted by others for common use.

Researchers at the National Cancer Institute (NCI) report of a full-length baculovirus-expressed LANA/ORF73 protein with 99% specificity and 84% sensitivity, though these data have failed to be published. Reproducibility of these numbers has been questionable because among the published seroepidemiology studies which report the use of this assay system, none

adequately describes the methods for performing the assay(153, 280, 441), and the overall utility of the assay has been brought into question by the group which has used it in the past. There is currently no intention to continue use of this serologic method in future seroepidemiology studies conducted at NCI due to problems with consistency (Personal communication, Denise Whitby).

As described in detail in chapter 2, we describe a novel recombinant LANA1-gst ELISA which provides a high degree of sensitivity and specificity when used in a combined format with the ORF65 and K8.1 lytic ELISA systems. In addition, further discussion of ELISA serological techniques for defining KSHV infection and the associated complications that have been observed are explored in the article.

1.4 The Epidemiology and Seroepidemiology of KSHV

In the following section, the epidemiology of KSHV is reviewed including transmission modalities, clinical course in various populations and treatment options for individuals infected. In addition, the chapter explores in depth the seroepidemiology of KSHV including, a summary of global KSHV infection rates, as defined by serological methods, and a discussion of the adequacy of the assays currently available for serological definition of KSHV infection.

1.4.1. KSHV Transmission

Though considerable efforts have been made to explain the routes of KSHV infection, the precise mode(s) of transmission have yet to be adequately elucidated. A number of potential routes of transmission are possible because KSHV has been isolated in a number of tissues and body fluids from infected individuals including blood, semen and prostate tissues(114, 199, 292, 332), the genital tract of women(442), uncommonly in rectal samples

(76) and urine(371) and most commonly in saliva and oral fluids(235, 330, 426).

Epidemiologically, sufficient evidence exists to support both sexual and nonsexual transmission modalities.

1.4.1.1 Sexual Transmission of KSHV

KSHV can be transmitted through sexual contact and in North America and Europe is likely the most important transmission pathway. Studies have shown that male homosexual transmission is the primary route; however, heterosexual transmission has been reported.

Homosexual Transmission

A number of studies demonstrate that among homosexual men KSHV transmission occurs via a sexual route(131, 141, 227, 275, 283, 310, 355), though the precise mechanism of transmission has not been fully established. Correlations have been demonstrated between specific sexual practices and increased KSHV seropositivity and include receptive anal intercourse, insertive anilingus, total number of sex partners, and deep kissing with an HIV infected partner(184, 275, 283, 310, 330). In addition, saliva as a lubricant for penetrative sex may be an important transmission route(78). Though these correlations have been noted, few studies have prospectively assessed sexual practices and KSHV seroconversion, making temporal conclusions with the current data not possible. Further confounding these associations is that multiple specific individual sexual practices are commonly practiced by individuals, making isolated inferences regarding a unique sexual transmission mode difficult to study. One study does report that orogenital receptive and insertive sex with more than 5 partners in 6 months was an independent risk factor for seroconversion(141). It is currently unclear whether the risk of KSHV infection is elevated or diminished among homosexual

women. Studies of homosexual female risk factors are required for an understanding of potential transmission pathways among this population.

Heterosexual Transmission

Definitive proof for heterosexual transmission is still lacking largely due to conflicting studies using different KSHV detection methods. However, the weight of evidence suggests that heterosexual transmission of KSHV is likely, though KSHV transmission efficiency via penile-vaginal sex is low. Studies of female commercial sex workers have shown that KSHV seroprevalence is higher among women with at least one other sexually transmitted disease, suggesting a sexual transmission route(148, 267). In one African study of rural families, women married to a KSHV positive man had a sevenfold risk of infection providing further evidence that KSHV is likely transmitted sexually between spouses(281). To date, studies show evidence of unidirectional male to female infection via a heterosexual route but currently evidence is lacking for conclusions to be made regarding female to male transmission through a sexual route.

1.4.1.2. Nonsexual Transmission of KSHV

Several studies suggest transmission via nonsexual contact is common in KSHV endemic areas such as Africa and the Mediterranean. Various lines of evidence support nonsexual transmission. For example, in endemic areas seroprevalence is elevated among children and increases with age to near adult levels prior to adolescence(14, 78, 177, 260, 279, 281, 316, 344, 383) and KSHV clustering within families and crowding have been observed as important risk factors for nonsexual transmission(16, 123, 344, 440). In addition, recent reports have demonstrated that infection through infected blood products and transplanted organs is possible.

KSHV transmission via close nonsexual contact

Mother-to-child transmission studies have been undertaken and can be loosely placed into three categories based upon their findings: (1) horizontal mother-to-child transmission occurs (59, 65, 190, 224, 260, 344, 377) and is associated with increasing maternal antibody titer level(125, 344, 394). (2) vertical mother-to-child transmission is unlikely or extremely rare(142), and (3) KSHV infected mothers can passively transmit antibodies to their infants who later serorevert(72, 78, 89, 177, 260).

In utero transmission has not been sufficiently demonstrated(180). However, some studies have found KSHV DNA present among newborn infants using PCR techniques, though infrequently(65, 263, 264). Transmission through breast milk is unlikely(64) though KSHV DNA was isolated in one study(125). Antibodies to KSHV have been isolated in breast milk but it is believed that these are passively transmitted and the probability that infectious virus is transmitted through this mechanism is at most very uncommon and would likely require an aggressive disseminated maternal infection with KSHV.

The mechanism by which nonsexual transmission occurs is not known with certainty. However, oral shedding of KSHV is the leading hypothesis and has been demonstrated as a mode of transmission(427). KSHV DNA copy number in saliva is higher than in other body fluids (48, 330). Taken together, the weight of evidence suggests KSHV is transmitted nonsexually in endemic populations likely through salivary contact. By whichever mechanism transmission occurs, horizontal transmission is the primary route of non-sexual KSHV infection with vertical transmission unlikely to occur, although definitive evidence is still lacking.

KSHV transmission via blood

Transmission through blood and blood products has been demonstrated though transmission efficiency via this route appears to be low. In the past, transmission of KSHV through blood products has been discounted due to the low rates of KS observed among hemophiliacs and IV drug users. However, increasing intravenous drug use was found to be associated with an increase in KSHV seroprevalence among a large cohort of women in the United States(77) and has also been found to be an independent risk factor for KSHV infection among men and women in international studies(38, 358, 359, 362, 398). These findings suggest needle-sharing and inter-person blood transfer is a possible mechanism for KSHV transmission.

Studies of KSHV among blood donors have shown that KSHV infected individuals do provide blood products for transfusion to others (28, 33, 47, 73, 99, 103, 123, 150, 152, 163, 171, 198, 202, 219, 236). Early on, some argued that transmission via this route was biologically plausible (152), though early studies based on small samples did not find evidence of transfusion-associated transmission(154, 319). The risk of becoming infected through infected blood products is not clear, however larger and more recent studies conducted in Africa estimate KSHV transmission risk to be around 3% per transfusion(154, 280).

A recent study conducted in the US demonstrates that transfusion-associated transmission is likely to occur and estimates the KSHV transmission risk per transfused component to be less than 0.1%(136). Though the effective transmission rate of infection is low, the number of transfusions per year is high. The American Association of Blood Banks estimates that approximately 5 million transfusions occur in the US each year(11). Based upon these estimates, up to 5,000 cases of transfusion-associated KSHV infections can be expected

to occur every year in the United States. Taken together the body of evidence supports blood transmission as a possible route of KSHV transmission but with low efficiency.

KSHV transmission via organ transplantation

Solid organ transplant recipients can acquire KSHV from infected donor organs(30, 256, 268, 288, 308, 326, 356). Though the exact viral transmission efficiency via this route is unknown, the probability of seronegative transplant recipients becoming infected with KSHV through infected renal allografts has been estimated to be 2%-12%(288, 356). One report of 28 cases of posttransplantation KS showed that 82% of KS cases were due to KSHV reactivation and 18% were due to new infection from the transplanted organ(256).

Additionally, recipients who become infected through one of the traditional transmission pathways post-transplantation are at increased risk for developing KS. It is currently unclear whether KS risk differs between recipients with denovo infection from an infected organ compared to those with viral reactivation or post-transplant primary infection.

1.5. KSHV Seroepidemiology

Largely as a result of seroepidemiology studies, much has been determined regarding the basic epidemiology of KSHV including modes of transmission, disease associations and pathological consequences of infection, including risks factors for developing KS.

Additionally, seroepidemiology studies of KSHV have categorized the global distribution of KSHV infection and have provided an understanding of clinical and behavioral risk factors that are associated with infection.

Though no gold standard assays exist for KSHV, certain trends are clear. For example, seroepidemiology studies have shown that KSHV is not a ubiquitous virus worldwide, as are most other herpesviruses. In addition, studies of KSHV prevalence suggest that KSHV

infection is persistent, as is EBV and HHV-6, as demonstrated through the correlation of prevalence with age(316). Current serological techniques have uncovered these phenomena and the sensitivity and specificity of these assays has not been a problem in elucidating these associations among some high risk populations. However, the utility of these assays in low-titer, low-risk populations is unclear. The largest discrepancies between KSHV assays and laboratory interpretation of assay results arise in the populations at lowest risk of developing KS. For example, a number of reports suggest the KSHV seroprevalence among the US blood donor population is likely less than 5%(333). However, others have postulated that a large proportion of the general population classified as uninfected by many serological assays and PCR techniques are actually asymptotically KSHV infected in low risk groups with titers below the level of detection of currently available assay systems.

Though serological assays and techniques have provided utility in exploring basic epidemiological and scientific questions regarding population infection rates, the current assay systems routinely used require refinement and fine tuning to go beyond the current knowledge base and to be of adequate and consistent clinical utility, especially in populations at low risk of infection.

1.5.1. Global Distribution of KSHV Infection

The geographical distribution of KSHV infection has not been completely defined, though in general it has been noted that the distribution of KSHV infection matches closely the global distribution of KS prevalence(142). Figure 1-7 shows KSHV prevalence worldwide based upon published reports.

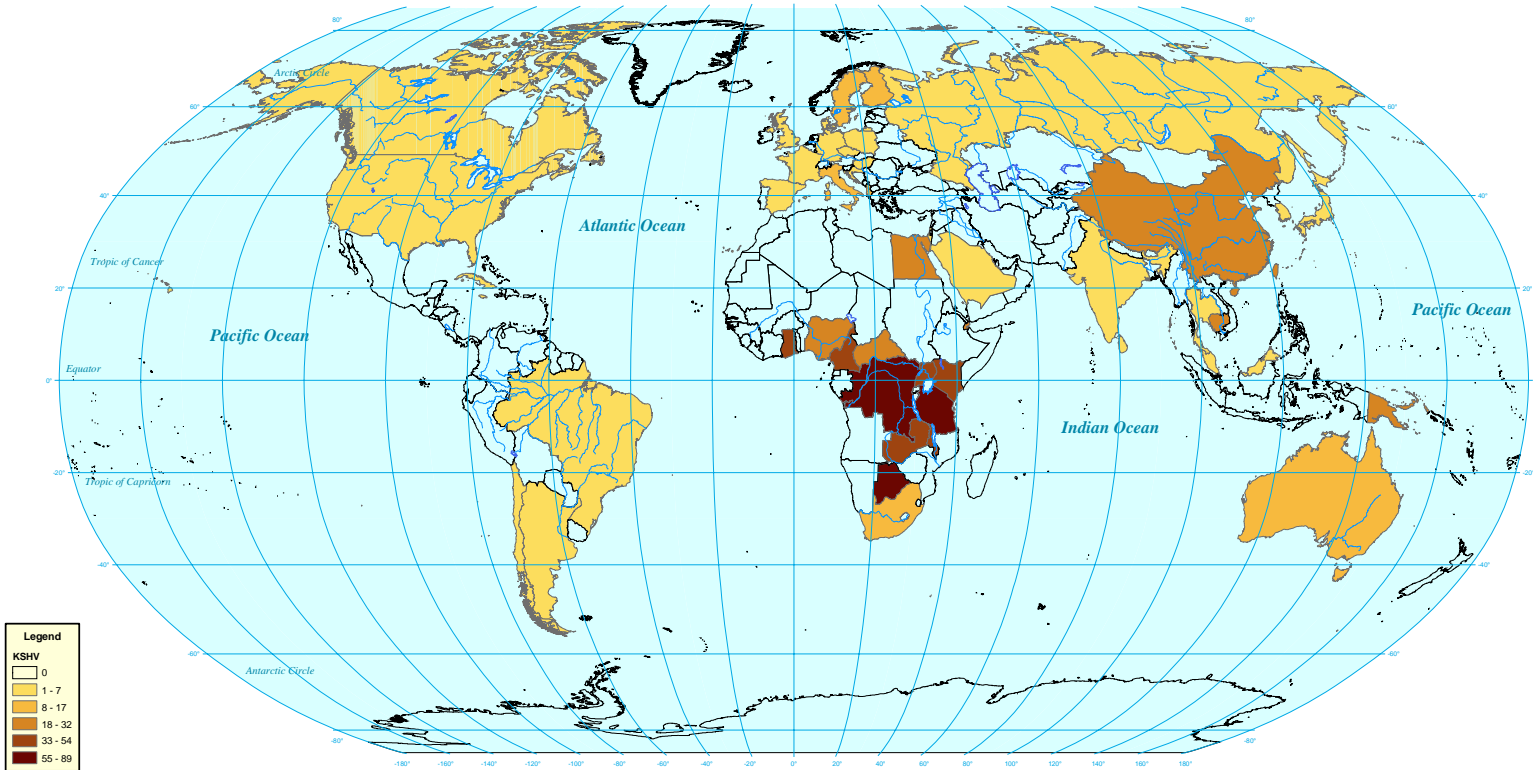


Figure 1-7. Global distribution of KSHV based on studies of KSHV prevalence. General population rates reported when available. Multiple studies done in same geographic location were averaged with weighting performed based upon sample size. It should be noted that a variety of different serological techniques were employed to obtain KSHV prevalence estimates. Prevalence estimates are presented as percents, a value of zero percent (clear areas) have no available data reported. Based upon references (1, 9, 26, 27, 33, 93, 104, 123, 124, 127, 134, 139, 140, 150, 155, 171, 185, 187, 198, 210, 212, 213, 218, 219, 226, 228, 236, 267, 270, 276, 281, 304, 309, 333, 339, 349, 354, 365, 374, 376, 382, 384, 391, 392, 401, 404, 407, 418, 428, 430, 431, 439, 451).

KSHV prevalence in sub-Saharan Africa is the highest in the world (>50%)(20, 27, 33, 102, 127, 142, 151, 155, 198, 224, 231, 260, 281, 309, 316, 328, 344, 384, 392, 401, 421, 443, 444), with intermediate prevalence throughout the Mediterranean (15-30%) (54, 56, 62, 63, 73, 93, 102, 123, 155, 174, 210-212, 294, 326, 361, 362, 384, 440, 451)and low prevalence throughout the rest of Europe (<5%)(152, 162, 171, 191, 276, 304, 347, 375, 404). KSHV prevalence also appears low in the Western hemisphere with most studies reporting seroprevalence estimates of less than five percent throughout North, South and Latin America(1, 28, 29, 102, 136, 174, 236, 276, 333, 339, 350, 452). However, recent reports demonstrate that among isolated populations in the Americas such as the Brazilian Amerindians, the potential for KSHV endemicity exists(46) with a reported prevalence of 53%. It may be that other isolated populations such as Inuit populations or other Native American populations may have elevated KSHV seroprevalence, though studies of these types have not been reported in the scientific literature.

It is important to note that categorization of nationwide KSHV prevalence based upon isolated seroepidemiological studies is simply an estimate. In some countries, such as the United States, it is likely that among the general population the reported prevalence of KSHV infection as less than 5% is accurate because numerous studies using multiple techniques have contributed to our understanding. However in other countries, such as China, prevalence estimates are based on a few local studies using serological tests that may not have the highest sensitivity or specificity. Our current understanding of the KSHV prevalence in Australia is based upon one unpublished report(139).

Additionally, through epidemiological studies, we understand that country boundaries can be arbitrary and populations within those boundaries may not be homogenous. For

instance, in Italy, seroprevalence estimates range from <10% in the North to >20% in the South. Also, KSHV infection can be concentrated in certain population groups who reside in countries with overall low prevalence rates. KSHV infection among homosexual males residing in the US may be 10 times or more higher than the rest of the population(211).

Studies of KSHV seroprevalence are not standardized and a meta-analysis of the numerous studies done on KSHV infection rates has not been undertaken. Because a gold-standard test for KSHV does not currently exist, multiple methods have been used to define KSHV infection within individuals. All of the current tests have strengths and weaknesses but some level of misclassification has been reported for every method described to date. These caveats should be noted when interpreting studies of worldwide KSHV prevalence. Though absolute precision of KSHV infection rates worldwide is unknown, in general it is accepted that Europe and the Americas are low prevalence regions, the Mediterranean moderate prevalence, and sub-Saharan Africa has the highest prevalence of KSHV worldwide.

1.5.2. Perceived Need for Improved Serological Methods for the Detection of KSHV

In an editorial commentary in the Journal of Infectious Diseases, David Hudnall points out some of the conflicts which arise from using different serological assays to measure KSHV prevalence in populations at low risk for developing KS(201). The primary question addressed is whether assay sensitivity or specificity is most important when both cannot be simultaneously achieved through the use of one assay. Specifically, Hudnall argues that immunofluorescence assays which target latent viral antigens are of use only in high risk groups and that through induction of lytic antigens in primary effusion cell lines prior to serological testing that test sensitivity can be increased. In fact, the prevalence of KSHV was found to be dramatically higher (23%-29%) using the lytic assay in three studies of healthy US

blood donors(202, 249, 350). This has led some to the conclusion that not only does the latent LANA1 IFA lack sensitivity but all other serological techniques, including ELISA assays targeting lytic viral epitopes present only during lytic activation, lack sensitivity as well.

An alternative explanation is that the lytic IFA, though highly sensitive, is of little utility in high or low risk populations due to its low specificity. The low specificity and lack of concordance with other serological assays has been well documented(333, 350, 375) and reviewed in(102, 378, 379). Because the debate continues as to the best method for defining KSHV seroprevalence many laboratories have chosen to work with KSHV assays with intermediate sensitivities and specificities. Additionally, many groups use multiple assays to increase overall diagnostic accuracy, moving away from individual assays at either end of the sensitivity/specificity spectrum.

Recently authors have directly pointed out the need for seroassays which target multiple epitopes(241) and others have made compelling arguments for the clinical need for accurate diagnosis of KSHV infection(296). Because the need for improved diagnostic assays for KSHV exist, and because there is no consensus as to which assays or combination of assays should be used to concurrently improve sensitivity and specificity, this research is designed to address these problems.

In chapter 2, research is presented which demonstrates a novel serological approach to improving the diagnostic capabilities of defining KSHV infection. Gold standard KSHV positive and negative sera were obtained and tested using multiple serological assays. In addition, the development and testing of a novel LANA1 ELISA assay is described. A new method for plating sera which improves diagnostic specificity is explained and upon optimization of the serological method the system is validated through testing of an

independent gold standard set of sera. Upon validation we conduct studies of KSHV seroprevalence among various populations at high and low risk of developing KS. In addition, we rigorously examine our new method through testing of sera known to be cross-reactive in other viral ELISA assays.

In chapter 3, we present a case-control study of primary pulmonary hypertension (PPH) and KSHV infection using assays described in chapter 2. The justification for undertaking this case-control study was two-fold. First, a previous report described a strong association between PPH and KSHV infection using PCR and IHC on lung tissue. A number of studies have shown these methods to be unreliable for diagnostic purposes and these methods have been responsible for a number of spurious associations with KSHV in the past. Second, the epidemiology of PPH and KSHV are seemingly at odds. Therefore, we used our serological assays to test whether KSHV seroprevalence was elevated among subjects with PPH compared to healthy controls.

In concluding the dissertation, a global discussion describing an overview of the research presented and specific strengths and weaknesses of the projects are then highlighted, ultimately concluding with the perceived impact of this work and what logical future work can be undertaken from the knowledge gained from this research.

2. Diagnosis of Kaposi's Sarcoma-Associated Herpesvirus (HHV-8) Infection Using a Multiantigen Detection Algorithm.

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2.1. Abstract

Though substantial efforts have been made, the ability to readily and accurately diagnose Kaposi's sarcoma-associated herpesvirus (KSHV, or HHV-8) in individuals remains a demanding task. Sensitivities and specificities of the available diagnostic methods widely range and, as a matter of practicality, many are inadequate for large-scale screening studies. We propose here a high-throughput serological algorithm for detecting KSHV in human sera with high sensitivity (93%-96%) and high specificity (94%-98%). This method is based on peptide based ELISAs directed against ORF K8.1 and ORF65 proteins and a newly developed KSHV latency associated nuclear antigen (LANA) ELISA based on purified recombinant antigen expressed in baculovirus. This antigen is expressed as a glutathione-S-transferase fusion protein which binds to glutathione-linked ELISA plates allowing the attachment of viral antigen to ELISA plates with high efficiency. The validated serological algorithm is then used to determine seroprevalence rates among populations of particular clinical and epidemiological importance. Among cadaveric renal transplant donors, from whom KSHV can be transmitted to uninfected recipients, we find a seroprevalence of 4.0% using a highly specific algorithm and 8.0% with a highly sensitive algorithm. Among patients requiring bone-marrow transplantation 3.0% tested positive with the specific algorithm and 10% were positive with the more sensitive algorithm. We also demonstrate that patients with autoimmune disorders are more likely to produce false-positive KSHV ELISA test results than blood donor controls. Because the need for high quality KSHV detection methods are warranted and because questions remain about the optimal methods for assessing KSHV infection in individuals, we propose a systematic approach to standardize and optimize the assessment of KSHV infection rates using established and novel serological assays and methods.

2.2. Introduction

Since the discovery of Kaposi's sarcoma-associated herpesvirus (KSHV)(100), also known as human herpesvirus 8 (HHV-8), the virus has been implicated in diseases of the immunocompromised host including Kaposi's sarcoma (KS), multicentric Castleman's disease and primary effusion lymphoma(295). In the US, among immunocompromised individuals who are KSHV infected, the life-time risk of developing KS is 20-30%(78, 165, 275, 310, 356). With a prevalence of 3-10%(78, 333) among iatrogenically immunosuppressed patients and 20-40% among HIV-infected individuals(141, 174, 179, 227, 324), KSHV is clearly of significant public health concern in the US. In regions of Africa where endemic infection can exceed 60%(343), the morbidity and mortality associated with KSHV related sequelae are of even greater concern.

Significant morbidity and mortality are associated with a diagnosis of KS among transplant recipients. In one study of liver transplant recipients, 100% of KSHV negative recipients who received a KSHV-infected allograft seroconverted within 6 months post-transplantation. Of those, 50% developed KS and died of multiorgan failure within 1 year post-transplantation (268).

Among kidney transplant recipients seropositive for KSHV prior to transplantation 23%-28% develop KS(92, 165). A similar rate has been reported among heart transplant recipients where 25% of KSHV positive recipients developed KS (149). In addition to infection prior to transplantation, recipients can acquire KSHV from infected donor organs(30, 256, 268, 288, 308, 326, 356). Though the exact viral transmission efficiency via this route is unknown, the probability of seronegative transplant recipients becoming infected with KSHV through infected renal allografts has been estimated to be 2%-12%(288, 356). In addition,

recipients of bone marrow transplants who are infected with KSHV infection are at increased risk of bone marrow failure. Regardless of whether the virus is acquired before or after immunosuppression, it is clear that both primary infection with and reactivation of KSHV dramatically increases the risk of malignant and nonmalignant disease among HIV-infected individuals and transplant recipients(137, 220, 255, 257, 259, 278).

A recent study conducted in the US demonstrates that transfusion-associated transmission is likely to occur and estimates the KSHV transmission risk per transfused component is around 0.1%(136). Though the effective transmission rate of infection is low, the number of transfusions per year is high. The American Association of Blood Banks estimates that approximately 5 million transfusions occur in the US each year(11). Based upon these estimates, up to 5,000 cases of transfusion-associated KSHV infections can be expected to occur every year in the United States.

Though the existence of KSHV has been recognized for over a decade(100) definitive diagnosis of viral infection remains a challenge. Some of the factors which make definitive diagnosis difficult include: a large proportion of KSHV infected individuals do not have detectable levels of KSHV DNA in peripheral blood (302), concordance between serological assays targeting different antibodies varies(437), and assembling a known uninfected cohort with 100% certainty is not possible(237, 274).

Serologic tests for KSHV were first developed for reactivity to the KSHV latency associated nuclear antigen (LANA)(173, 174, 301). These assays were performed by indirect immunofluorescence (IFA)(227) or western blot assays using infected cell lines(173). While the LANA IFA is highly specific for KSHV infection, its sensitivity is only approximately 80%,(174) resulting in high rates of false-negativity. Further, IFAs are cumbersome and

cannot be used for high-throughput screening, disadvantages that apply to an even larger extent for Western blot assays.

In addition to the LANA IFA, other serologic tests have been developed against KSHV lytic antigens including enzyme-linked immunosorbent assays (ELISA) targeting structural antigens. Unlike latent antigens, structural antigens are highly conserved among herpesviruses resulting in substantial cross-reactivity and high false positivity rates(249). Whole cell lytic antigen assays measure KSHV seroreactivity, but this seroreactivity can also be partially absorbed by non-KSHV infected cell lysates demonstrating that seroreactivity to whole cell lytic antigens can be nonspecific(174, 249, 301). Attempts have been made to produce a recombinant LANA1 protein (rLANA1), but marked loss of sensitivity has been noted(318).

One approach to increase assay specificity for KSHV lytic antigens has been to use recombinant protein ELISAs. Both whole structural antigens and immunodominant peptides have been evaluated in an ELISA format(375, 400). These tests have improved sensitivity and specificity characteristics compared to the whole cell lytic IFA(115, 156, 209, 333) though at present, no single recombinant antigen assay has sufficient sensitivity/specificity characteristics for use in routine clinical screening. Optimal detection of KSHV seropositivity appears to require use of both latent and lytic antigens which are independently screened and scored for seroreactivity.

Because of the need for high quality KSHV detection methods, we used a systematic approach to standardize and optimize the assessment of KSHV infection rates using established and novel serological assays and methods. Upon validating our assay algorithm we examined seroprevalence rates among high-risk populations of clinical interest including transplant donors and patients with autoimmune disease who may generate false positive results.

2.3. Materials and Methods

2.3.1. Subjects

Initial assay evaluation was performed on 90 case patient sera obtained from persons with biopsy-confirmed KS. Sera were drawn within 12 months after KS diagnosis and all case subjects were HIV infected males with a diagnosis of AIDS. Case sera were collected from May of 1990 to May of 2004. Control sera (100) were obtained from New York City Blood Bank (NYCBB) donors, all of whom had previously been screened and found to be LANA IFA seronegative, as well as negative for HIV, HCV, HBV, syphilis and HTLV1. Control sera were collected throughout 1991. A second independent set of case and control sera were tested for purposes of validating our assay algorithm. Case sera for the independent test set, a total of 87, were obtained from two large longitudinal studies of HIV-infected men (241, 381) and defined as sera drawn from subjects after a clinical diagnosis of KS. These case sera were collected from 1985 to 2002. Control sera for the test set were an additional 100 blood donors from the NYCBB.

Sera from patients with severe aplastic anemia and myelodysplastic syndrome, collected from 1992-2000, were obtained from the National Marrow Donor Program (NMDP) and matched with respect to age, gender and history of blood transfusion. Screened cadaveric renal transplant donor sera were obtained from the Center for Organ Recovery and Education (CORE), Pittsburgh Pennsylvania.

Sera from patients with systemic lupus erythematosus (SLE) (5 male, 45 female) were obtained from the University of Pittsburgh Division of Rheumatology and Clinical Immunology and 50 blood donor controls were obtained from the NYC blood bank. Sera from SLE patients were collected from 2001-2003. Prior to KSHV testing, case and control

specimens, as well as 5 positive laboratory controls from the University of Pittsburgh Cancer Institute (UPCI) were blinded. Informed consent from all study participants and IRB approval were obtained in accordance with the guidelines for human experimentation of the University of Pittsburgh.

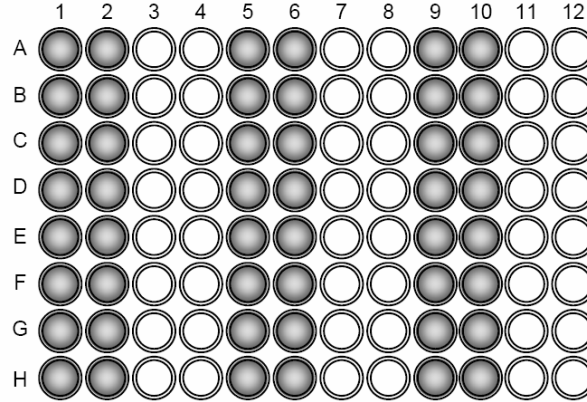
2.3.2. Serological testing

Sera were tested by using ELISA serological assays against recombinant baculovirus expressed LANA1 and against peptide-based ELISAs for ORF65 and K8.1 antigens. In addition, sera from patients with SLE were tested using a LANA1 immunofluorescence assay (IFA). For the ELISA assays, serum at a dilution of 1:100, was added to 4 wells: two coated with peptide (5 µg/µl) and the remaining 2 wells without peptide. Optical density (OD) values were read on a Dynatech Laboratories (Chantilly, VA) MRX 1CXA0716 plate reader at 405nm wavelength after reaction with rabbit anti-human IgG HRP (1:6000, DAKO, Carpinteria, CA) and development in 3,3',5,5'-tetramethylbenzidine (BioRad, Hercules, CA) .

ODs for a given sample were calculated as the mean OD of the wells containing peptide minus the mean OD of the wells containing no peptide (see Figure 1). All samples in the study were each independently tested in duplicate and all serological testing was done in a blinded fashion. Blinding was maintained until the completion of all serological testing.

Figure 2-1. Enzyme-Linked Immunosorbent Assay (ELISA) plating scheme.

Each sample tested has two wells coated with antigen (indicated by gray shading in columns 1,2,5,6,9 and 10) and two wells that are not coated with antigen (unshaded circles of columns 3,4,7,8,11 and 12). The average of the non-coated wells, used as a background correction, is subtracted from the average of the peptide-coated wells to give the adjusted optical density.



$$\text{Adjusted OD} = \left[\frac{\text{Gray Well} + \text{Gray Well}}{2} \right] - \left[\frac{\text{White Well} + \text{White Well}}{2} \right]$$

2.3.3. K8.1 and ORF65 peptide ELISAs

Antibody testing was performed using enzyme-linked immunosorbent assays (ELISA) based on peptides from the open reading frames (ORF) 65 and K8.1 with sequences; ASDILTTLSSTTETAAPAVADARKPPSGKKK and RSHLGFWQEGWSGQVYQDWLGRMNCSYENMT respectively as previously described (329, 400) with the modifications noted above.

2.3.4. rLANA1-GST ELISA

ORF73 recombinant baculovirus was produced via transfection with a pDEST20 plasmid vector (Invitrogen, Carlsbad, CA) containing GST-fused full length LANA1 protein. Sf9 cells were grown in BacPak complete medium (Clontech BD-Biosciences, Palo Alto, California) on a shaker at 135 rpm in a 27°C incubator. At the time of infection, the concentration of cells was adjusted to 1×10^6 cells/ml, spun down, and resuspended in 1/10 of

the total volume of unsupplemented medium. Baculovirus was used to infect the cells at a multiplicity of infection equal to one for 45 minutes at room temperature. Supplemented BacPak medium was added to full volume and the infected cells were incubated for 3 days at 27°C prior to harvest. The cells were pelleted, frozen in an ethanol-dry ice bath and thawed in a solution of 10mM TrisHCl pH7.5, 130mM NaCl, 10mM NaF, 10mM sodium phosphate, 10mM sodium pyrophosphate, and 1% Triton X-100 with protease inhibitors Aprotinin (Roche #236 624), Leupeptin (Roche #1 017 101), Pepstatin (Roche #1 359 053), and PMSF (Sigma-Aldrich, St. Louis, MO) added just prior to use. The lysed cells were spun down and the supernatant was collected and purified using 1ml glutathione sepharose beads (Amersham, Piscataway, NJ) per 5ml of cell lysate. The purified GST-LANA1 was eluted with a solution of 20mM glutathione (Sigma-Aldrich, St. Louis, MO) in 50mM Tris pH 8.0, in three fractions of 1ml each. The first two fractions were combined into a pooled stock with a final concentration of 0.4ug/ul.

Reacti-bind glutathione plates were used according to the manufacturer-provided protocol (Pierce, Rockford, IL). Plates were washed 3 times (200ul/well) with 1X PBS/0.05% Tween 20. Purified GST-LANA1 protein was diluted in wash buffer (0.018ug/ul) and added to the wells at a volume of 110ul. Plates were covered and left to incubate at room temperature for 1 hour and washed 3 times with 200ul wash buffer per well.

To measure anti-LANA1 antibodies, serum was added at a 1:100 dilution in 5% milk buffer, covered and incubated 1 hour at room temperature. Rabbit anti-human IgG HRP-conjugated antibody was diluted 1:6000 in 5% milk buffer and 100ul/well was added and incubated for 1 hour at room temperature. A colorimetric detection was performed as described for the peptide ELISAs.

2.3.5. LANA1 Immunofluorescence Assay

The LANA1 IFA was performed as previously described(174, 240). Two independent readers scored each sample. Two discordants occurred and were resolved by a third reader.

2.4. Results

2.4.1. Recombinant LANA1-GST ELISA Development and Optimization

Previous efforts to develop a recombinant LANA1 ELISA (rL) have been plagued by low test sensitivity. Initially, Olsen et al. mapped ORF73 (LANA1) for peptides which could potentially be used in an ELISA format but the sensitivities of the products were low (11%-44%)(318). Using the Semliki Forest virus system, Corchero et al. produced KSHV LANA for use in an ELISA format(115) though they found that it was of less utility than lytic assays.

We hypothesized that this low sensitivity may be in part to poor retention of antigen on plates due to the unusual charge structure of the LANA protein. To address this possibility, we generated a baculovirus-expressed LANA1-GST (rL-G) fusion protein which was coated to glutathione-linked 96-well plates. Using a mouse monoclonal antibody to LANA1, GST-LANA1 retention on glutathione plates was found to be approximately 20-30% greater than standard ELISA plates (data not shown). Increased seroreactivity is seen when patient sera were examined (Figure 2).

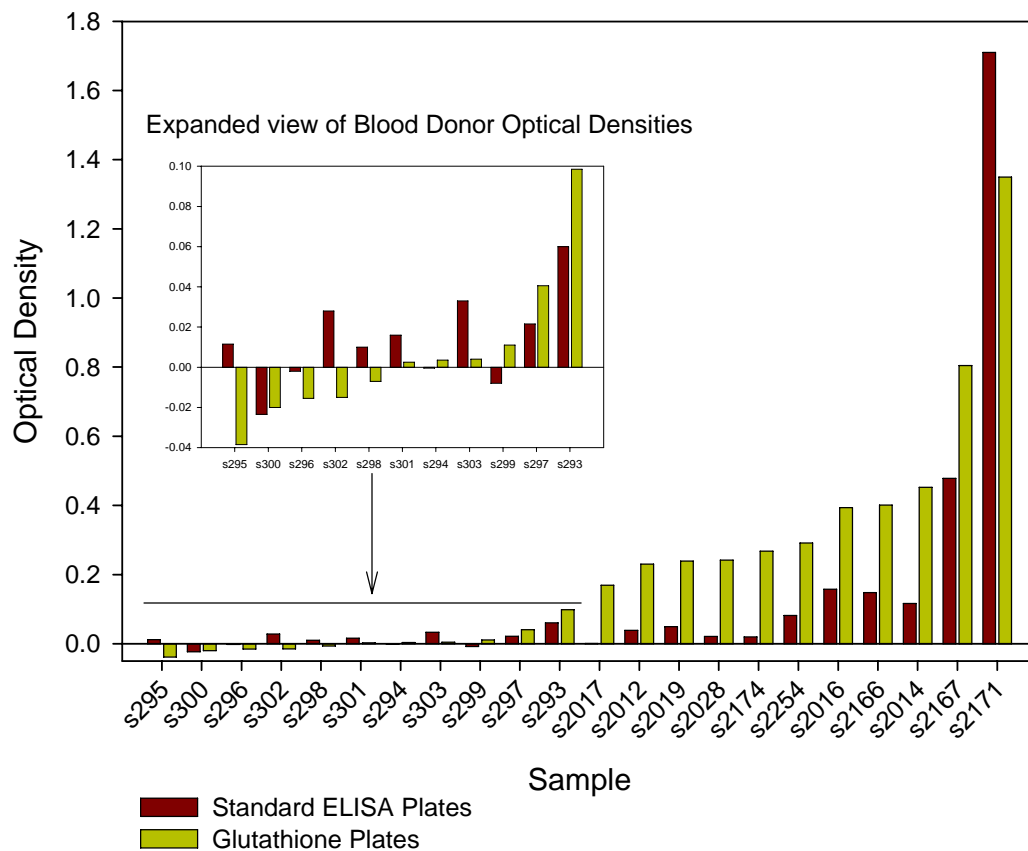


Figure 2-2. Optical density values – sera from patients infected and uninfected with KSHV plated on glutathione ELISA plates versus standard ELISA plates.

In all but one KS sera, OD values are higher on the glutathione plates, while no significant increase in reactivity is observed among the control sera. Using dilutions of rL-G among seroreactive and non-reactive serum samples, 1.0µg was found to give the maximum seroreactivity among the case sera with no appreciable reactivity observed among the control sera (Figure 3).

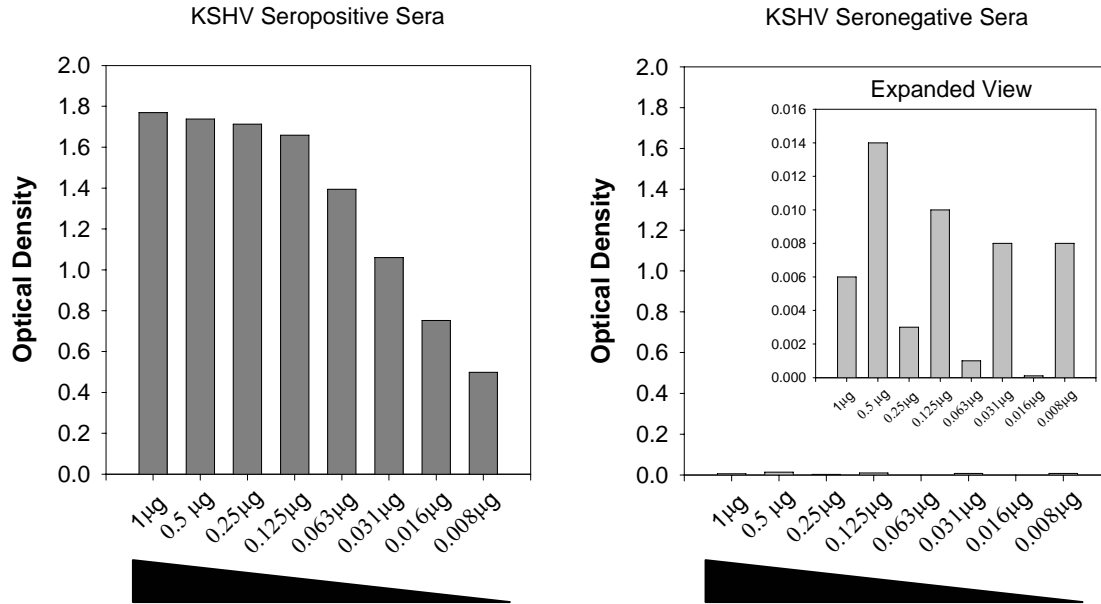


Figure 2-3. Optical density values observed from patients infected and uninfected with KSHV at differing concentrations of LANA1. The final concentration used was 1 µg/well.

2.4.2. KSHV Multi-antigen Algorithm

The rLG ELISA was evaluated together with the previously developed ORF65 and K8.1 ELISAs(400). In this analysis, 90 KS patient sera were compared to 100 blood donor sera. As seen in figure 4, case and control sera show significant overlap in optical densities for each of the three individual antigens. The rL-G ELISA shows highest specificity at very low OD values, while the K8.1 assay is the best discriminator overall when assessing the assays independently.

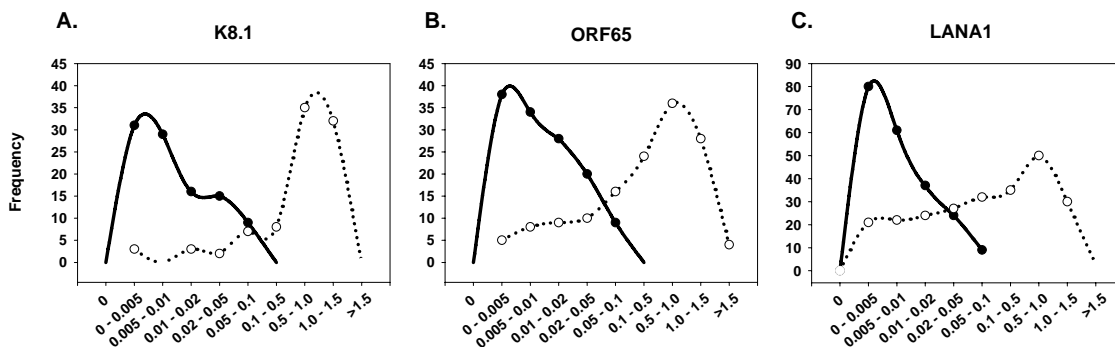
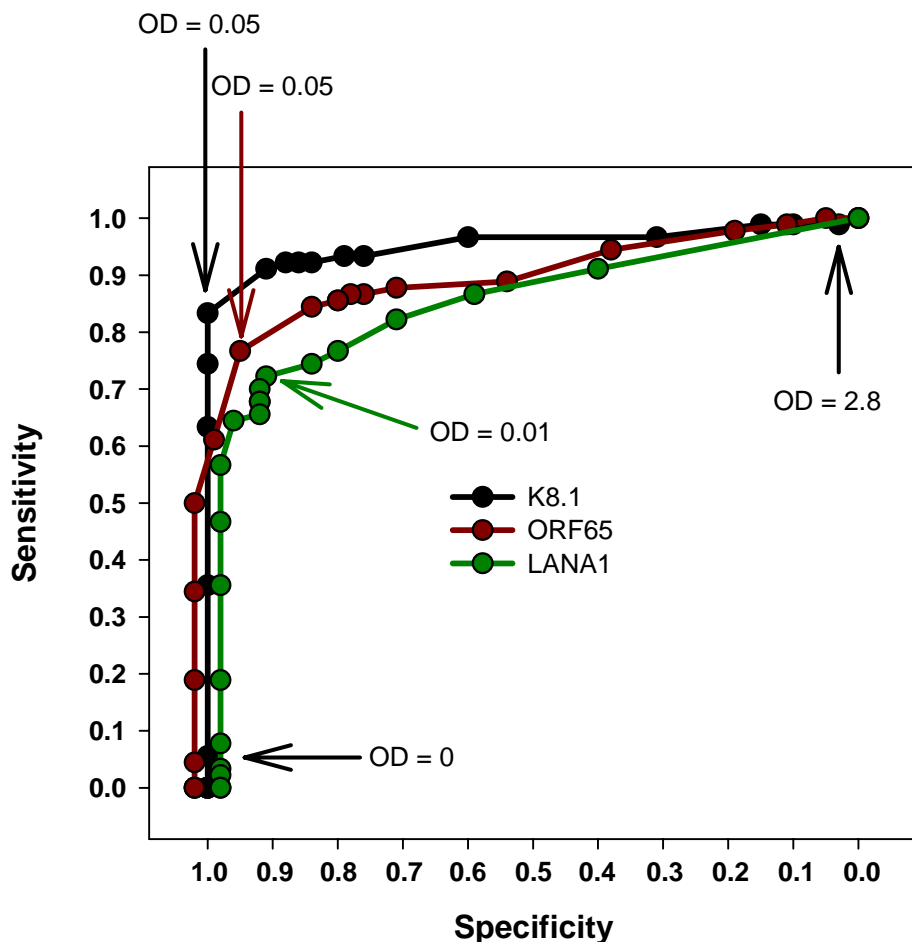


Figure 2-4. Distribution of optical density values among blood donors (closed circles) and subjects with Kaposi's sarcoma (open circles) for K8.1 (panel A), ORF65 (panel B), and LANA1 (panel C) enzyme-linked immunosorbent assays.

Figure 2-5. Receiver Operator Characteristic (ROC) curve of individual assay performance.
 Symbols indicate sensitivity and specificity of assays at differing optical density values.



To determine the optimal discriminator cut-off value for each assay alone, the data were plotted in a receiver operator characteristic curve (Figure 5). Based on the results of individual assays, we performed multiple iterations of OD cut-off values to obtain two algorithm models: one that maximizes sensitivity and one that maximizes specificity, while not allowing either value to drop below 90%.

Based upon these calculations a specimen was classified as positive if either the K8.1 assay OD was greater than 0.045, the ORF65 was greater than 0.0573 or LANA1 OD was greater than 0.041. This algorithm yielded a sensitivity of 95.6% and a specificity of 94.0%

(Table 1, panel A). For the high specificity algorithm, a model was chosen where specimens were classified as positive if either the K8.1 assay OD was greater than 0.045, the ORF65 was greater than 0.155 or LANA1 OD was greater than 0.048. This algorithm yielded a sensitivity of 93.3% and a specificity of 98.0% (Table 1, panel B).

Table 2-1. Training set contingency table and multi-assay sensitivity and specificity.

A. High Sensitivity Algorithm

Test Result	True Status		
	KS+	BD	
	KS+	BD	
KSHV+	86	6	92
KSHV –	4	94	98
	90	100	190

$$\text{Sensitivity} = \frac{86}{90} = 0.956$$

$$\text{Specificity} = \frac{94}{100} = 0.94$$

B. High Specificity Algorithm

Test Result	True Status		
	KS+	BD	
	KS+	BD	
KSHV+	84	2	86
KSHV –	6	98	104
	90	100	190

$$\text{Sensitivity} = \frac{84}{90} = 0.933$$

$$\text{Specificity} = \frac{98}{100} = 0.98$$

Panel A. values based on serological algorithm where a specimen is classified as positive if the optical density is greater than 0.045 for the K8.1 assay or >0.0573 for the ORF65 assay or >0.041 for the LANA1 assay. Panel B. values based on serological algorithm where a specimen is classified as positive if the optical density is greater than 0.045 for the K8.1 assay or >0.155 for the ORF65 assay or >0.048 for the LANA1 assay.

To validate the multi-assay algorithms, an independent test set of case and control sera were evaluated (Figure 6). Among the 100 new blood donor sera we observed a specificity of 94% and 97% with the sensitive and specific algorithms respectively. Among the 87 KS patient sera, the sensitivity dropped to 85.1% using the highly specific algorithm but remained at 93.1% with the more sensitive model.

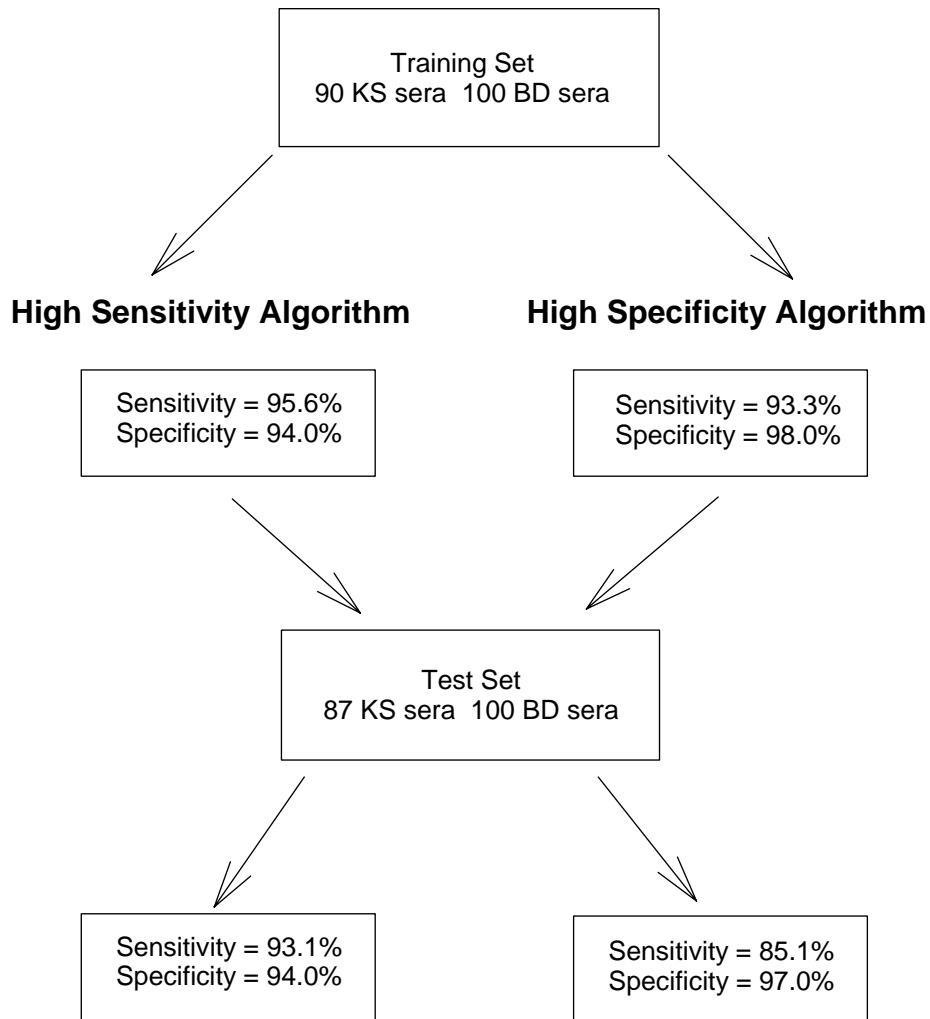


Figure 2-6. Selection Method for Determination of High Sensitivity and High Specificity Assay Algorithms.

To evaluate the rLG, ORF65 and K8.1 ELISAs, and because specimens were received from multiple repositories, we examined the integrity of the sera subjected to multiple freeze-thaws (Figure 7). All assays showed a statistically significant linear decrease in optical density (OD) with repeated freeze-thaws ($P < 0.01$). The total percentage decrease in signal intensity from the first measurement to the last was 13.6% for the ORF65 assay, 25.2% for LANA1, and 60.0% for K8.1, suggesting that handling can affect KSHV assay performance, but under most

conditions with proper handling, freeze-thaw cycles are unlikely to account for significant loss of seroreactivity.

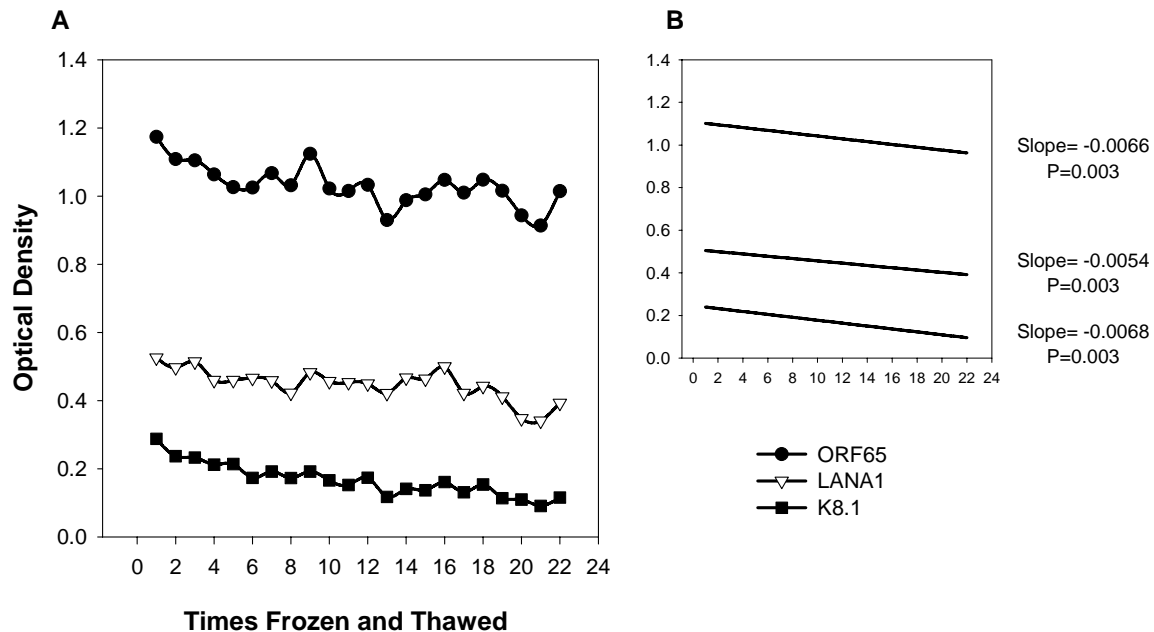


Figure 2-7. The effects of multiple freeze thaws on a human sero-reactive serum sample. Panel A. One patient sera seroreactive for ORF65, LANA1 and K8.1 was frozen and thawed a total of 22 times and tested on each assay. Panel B. Linear Regression of samples from panel A.

2.4.3. Serosurvey of Cadaveric Renal Transplant Donors

Because transmission of KSHV can occur from an infected kidney donor to an uninfected recipient, the optimized assay algorithms were used to test 50 screened sera from US renal transplant donors. Using the highly specific algorithm, overall seroprevalence was low at 4.0% (2/50) similar to the 2.8% (7/250) observed among US blood donors tested in this study ($P=0.46$) (Table 2). The odds ratio (OR) associated with this comparison is 1.4 and the 95% confidence interval (CI) ranges from 0.14 to 7.9. The more specific algorithm identified 8.0% (4/50) of the kidney donors as KSHV infected, though when compared to the 6.4% (16/250) found among blood donors with this method, no significant difference was observed between the groups ($P=0.43$; $OR=1.3$; $CI=0.41,4.0$).

Table 2-2. Comparison of seroprevalence of KSHV in different populations with a high sensitivity algorithm versus a high specificity algorithm

Population	Sample Size	High Sensitivity Algorithm		High Specificity Algorithm	
		Seroprevalence	P-value	Seroprevalence	P-value
Blood Donors – Training Set	100	6.0%	-----	2.0%	-----
Blood Donors – Test Set	100	6.0%	0.62	3.0%	0.50
Blood Donors – SLE Controls	50	8.0%	0.43	4.0%	0.41
Cadaveric Renal Transplant Donors	50	8.0%	0.43	4.0%	0.41
Severe Aplastic Anemia	50	10.0%	0.27	4.0%	0.41
Myelodysplastic Syndrome	50	10.0%	0.27	2.0%	0.71
Systemic Lupus Erythematosus	50	24.0%	0.002	20.0%	0.0003
KS Patients (Training set)	90	95.6%	----	93.3%	-----
KS Patients (Test set)	87	93.1%	0.35	85.1%	0.06

Note: P-values are Fishers exact and computed with US blood donors in training set as the referent group for low risk populations and subjects with KS in the training set as the referent group for comparisons in the high risk populations.

2.4.4. KSHV Case-Control Study of Patients with Myelodysplastic Syndrome and Severe

Aplastic Anemia

Among subjects requiring bone marrow transplantation the overall seroprevalence was 3.0% for the more specific model and 10.0% for the sensitive model and did not significantly differ from that observed among blood donors (P=0.4) (Table 2). In addition, KSHV

seroprevalence did not significantly differ between patients with severe aplastic anemia (4%) and patients with myelodysplastic syndrome (2%) using the more specific method (OR=2.0; 95% CI=0.10 - 122.9; and P=0.50) or with the more sensitive method, where both groups had an equivalent KSHV seroprevalence of 10%.

2.4.5. Case-Control Study of Patients with SLE and Blood Donor Controls

In using a case-control study design among 50 patients with SLE and 50 blood donors, our study objectives were two-fold; first, to rigorously test the utility of our assay system using a patient population known to produce false positive viral ELISA tests, and second to accurately identify the rate of KSHV infection among patients at increased risk of being on a course of immunosuppressive therapy, an important risk factor for KS. Our *a priori* null hypothesis was that KSHV seroprevalence among subjects with SLE and blood donor controls would be equivalent. However, using our ELISA assays, we observed a significantly higher KSHV seroprevalence among the SLE patients using the more specific algorithm (OR=6.0; 95% CI, 1.2-29.0) and the more sensitive algorithm (OR=3.6; 95% CI, 1.1-12.2).

Median OD values among SLE patients were significantly elevated compared to blood donors for the LANA1 assay (p=0.02) and the K8.1 assay (p=0.01). This trend was observed for ORF65 but did not reach a level of statistical significance (p=0.18). Based upon our more specific ELISA serological algorithm, of the 10 positive SLE samples, none were ORF65 positive, four were K8.1 positive only, six were LANA1 positive only, and one specimen was classified positive by K8.1 and LANA1. To further explore the apparent KSHV seroreactivity among patients with SLE, the LANA-1 immunofluorescence assay (IFA) was utilized.

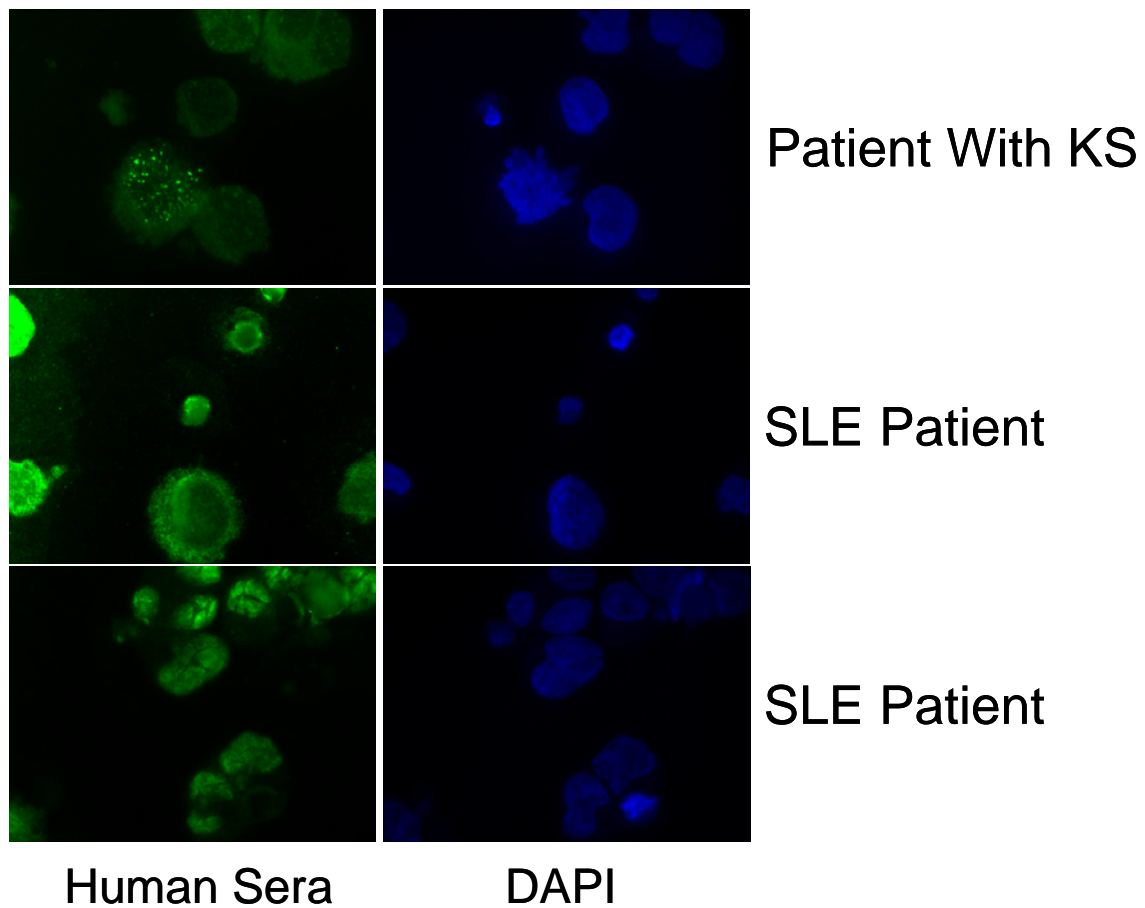


Figure 2-8. Representative IFA examples from KSHV-infected subject with KS and from two SLE patients with seroreactive ELISA results.

Figure 2-8 shows representative IFA examples from a KSHV-infected individual with KS and from two SLE patients. Note the increased level of background staining among the SLE patients but the lack of specific nuclear staining, indicative of KSHV positivity, which is observed in the positive control sample.

2.5. Discussion

Past reports have suggested that KSHV testing using multiple serological assays(274) or seroassays which target multiple epitopes(241) are needed to achieve maximal sensitivity. We have demonstrated the potential to increase KSHV detection accuracy rates beyond what has been previously reported through the combined use of established and novel assays.

In conducting this research we demonstrate the ability to target a KSHV latent viral antigen in an ELISA format. We utilized a glutathione-s-transferase tag and glutathione ELISA plates which allowed for the attachment of our recombinant LANA1 protein to the plates with high efficiency. As an independent assay, the sensitivity and specificity of the rL-G ELISA is similar to that observed for the LANA1 IFA but with significantly higher throughput. In addition, as one component of a multi-assay algorithm, the assay provides unique information which increases sensitivity beyond what is observed using an independent lytic-based ELISA.

By making minor adjustments in the OD cut-off values in the multi-assay algorithm we are able to present our results in comparative models that favor sensitivity or specificity. We have demonstrated that the relations in the case-control studies remain relatively constant even with minor changes in assay sensitivity and specificity (based upon p-values and odds ratios). Though the more sensitive algorithm definition consistently identified more seropositives, no significant difference in the ratio of positives to negatives was observed between the two different systems for any study population.

For screening efforts high sensitivity is often desired, however for an assay to provide clinical utility, high specificity must be achieved. The need for clinical testing of KSHV has been addressed in the past (286, 296) and the KSHV algorithms proposed here achieve this

high level of specificity (94%-98%). Among all US blood donors in the study 2.8% were observed to be KSHV infected with the algorithm designed for higher specificity and 6.0% when evaluated with the algorithm designed for higher sensitivity. It should be noted that an absolute evaluation of assay specificity is not possible when using US blood donors as a gold-standard negative population. US blood donor prevalence has been estimated to be 2-4%(333), therefore it can be reasonably expected that some proportion of the 2-6% of seropositive blood donors observed in this study are actually infected with KSHV, which could increase the overall specificity of both algorithms.

Epidemiologically it is somewhat implausible to suppose that KSHV infection is three to six times greater among patients with SLE compared to blood donors. We have provided evidence here of the potential that exists for false positive KSHV tests among SLE patients. As an alternative to the ELISA tests, we utilized a LANA1 IFA which visibly revealed the increase in non-specific antibody reactivity. Though the LANA1 IFA may be impractical for large-scale population screening, it can be reliably expected to produce accurate results among patient groups at increased risk of producing erroneous results among standard KSHV ELISA tests. Therefore, medical screening which assesses factors such as whether a patient has SLE or perhaps other autoimmune disorder can be used as one component in determining the type of KSHV testing algorithm that would best increase KSHV diagnostic specificity.

In conclusion, we describe a novel serological approach for defining KSHV seropositivity which has high sensitivity and specificity. Additionally, we present KSHV seroprevalence estimates of populations of particular clinical importance. Substantial evidence exists to suggest that KSHV can be transmitted to low risk populations through blood transfusion(47, 136) and organ transplantation (254) where a proportion will go on to develop

KSHV-related morbidity and mortality. KSHV screening of transplant donors and recipients could potentially prevent cases of KS from developing, aid in earlier diagnoses of KS that do develop, reduce non-KS morbidities associated with KSHV, and potentially block one of the viral transmission pathways of KSHV. The serological methods presented here may provide a mechanism to address these issues in the future.

3. **Kaposi sarcoma associated herpes virus and primary and secondary pulmonary hypertension.**

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3.1. Abstract

Background: Kaposi's sarcoma-associated herpesvirus (KSHV) has been implicated as a factor in the pathogenesis of primary pulmonary hypertension (PPH). We conducted a case-control study of patients with PPH and pulmonary hypertension associated with other disorders (secondary PH) to look for evidence of KSHV infection.

Materials and methods: The study population was comprised of patients diagnosed with pulmonary hypertension at the University of California San Francisco (UCSF) Medical Center Department of Cardiology between July and November 2003. Serological testing for KSHV was performed using enzyme-linked immunosorbent assays (ELISA) based on peptides from the open reading frames (ORF) 65 and K8.1, using sera from 19 patients diagnosed with PPH, 29 patients with secondary PH, and 150 control subjects

Results: The overall seroprevalence of KSHV among all study participants was 2.0%. The rate among controls was 0.7% (1/150), and among the study participants with PPH we found no evidence of KSHV infection (0/19). There was no significant difference between the observed seroprevalence of KSHV among subjects with PPH compared to controls ($P=0.89$). Of the 29 subjects diagnosed with secondary PH, 3 (10.3%) were KSHV seropositive. Significantly, 2 of the 3 KSHV-infected secondary PH patients were also HIV positive – which is a known independent risk factor for both KSHV infection and secondary PH.

Conclusion: Our data do not support KSHV infection as playing a significant role in PPH or non-HIV-associated secondary PH compared to age and gender matched control subjects.

Keywords: Pulmonary hypertension, Kaposi's sarcoma-associated herpesvirus, KSHV, HHV-8, K8.1, ORF65, ELISA

Abbreviations:

COPD: Chronic obstructive pulmonary disease

ELISA: Enzyme-linked immunosorbent assay

HHV-8: Human herpesvirus 8

HIV: Human immunodeficiency virus

IFA: Immunofluorescence assay

IHC: Immunohistochemistry

KSHV: Kaposi's sarcoma-associated herpesvirus

MCD: Multicentric Castleman's disease

OD: Optical density

ORF: Open reading frame

PCR: Polymerase chain reaction

PEL: Primary effusion lymphoma

PH: Pulmonary hypertension

PPH: Primary pulmonary hypertension

UCSF: University of California San-Francisco

UPCI: University of Pittsburgh Cancer Institute

3.2. Introduction

Pulmonary hypertension (PH) is a progressive disorder characterized by elevated mean pulmonary-artery pressure which may result in right ventricular failure. Primary pulmonary hypertension (PPH) is idiopathic, whereas secondary PH results from other conditions known to increase pulmonary artery pressure.(48, 369) PPH is most prevalent in persons 20 to 40 years of age and is 3 times more frequent in women than in men. This has led some to hypothesize that hormonal dynamics or an X-linked genetic locus may be involved with disease development.(253) More recently, it has been reported that infection with Kaposi sarcoma associated herpesvirus (KSHV), also known as human herpes virus type 8 (HHV-8), is associated with PPH.(110) That an infectious agent may play an etiologic role in this heretofore idiopathic disorder is an intriguing hypothesis that has potentially profound implications for its diagnosis, prevention, and treatment.

Since the discovery of KSHV in 1994,(100) a number of diseases and disorders have been hypothesized to be associated with KSHV. However to date, scientific consensus as to the diseases in which KSHV plays an etiologic role are limited to Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and a subset of multicentric Castleman's disease (MCD).(380) Epidemiologic studies have described potential modes of viral transmission,(77, 275, 330, 344, 356) rates of infection in different populations(1, 49, 99, 152, 211, 213, 227) and demographic and social risk factors associated with KSHV.(24, 27, 49, 77, 83, 118) Because KSHV is a relatively new virus, much is still unknown regarding its role as a potential etiologic agent or cofactor in diseases other than KS, PEL, and MCD. It is plausible that a number of rare idiopathic disease states could have a viral etiology or be influenced by a viral agent such as KSHV.

As the reported association between PPH and KSHV has been questioned,(193) we sought to examine whether an association exists between PPH and secondary PH and KSHV infection in a robust manner, using different methods than those previously employed. We designed a matched case-control study with sufficient power to examine whether KSHV infection differed between individuals with and without PH using highly specific and sensitive serological assays based on both lytic and latent KSHV antigens.

3.3 Materials and Methods

3.3.1. Subjects

The study population was comprised of patients diagnosed with pulmonary hypertension at the University of California San Francisco (UCSF) Medical Center Department of Cardiology between July and November 2003. Pulmonary hypertension was defined as right heart catheterization by a mean pulmonary artery pressure greater than 25 mmHg at rest. To elucidate the etiology of the PH and segregate patients to the PPH group and the secondary PH group, patients underwent diagnostic studies including echocardiography, ventilation-perfusion scans, pulmonary function testing, and blood sampling to evaluate for the presence of collagen vascular disease, liver disease, thyroid disease, and HIV. High resolution CT scanning, sleep studies, and coronary angiography were performed as indicated by the history and physical exam.

The PPH group included patients with idiopathic pulmonary hypertension without a demonstrable cause or association. The secondary PH group was comprised of all other patients who met the above definition for PH. In total, the study population included 19 subjects with PPH, 29 subjects with secondary PH (Table 3-1) and 150 controls (130 healthy

individuals, 19 with coronary artery disease and 1 with meningioma). Controls were matched to cases with respect to gender and age (+/- 5 years).

Prior to patient selection, sample size calculations were performed to establish the minimum sample size required to observe a significant difference in infection rates between cases and controls. Assuming the KSHV infection rate among patients with PPH was 32%, half the rate previously reported,(110) and the rate among controls was 3.3%, the rate of KSHV seroprevalence observed among US blood donors,(333) a minimum sample size of 19 cases and 57 controls should allow a significant effect to be observed between cases and controls with 95% confidence and 80% power. If the actual rate of KSHV infection among patients with PPH is 63% as reported by Cool et al., then our sample size should allow a significant effect to be observed between cases and controls with 99% confidence and 99% power.

Blood was drawn from each subject and serum was separated and frozen at -70° Celsius. Prior to KSHV testing, case and control specimens, as well as 10 positive and 10 negative laboratory controls from the University of Pittsburgh Cancer Institute (UPCI) were blinded. The blinding was performed and maintained by UCSF until the completion of all serological testing at UPCI. Informed consent was obtained from all study participants and IRB approval obtained in accordance with the guidelines for human experimentation of the University of California San Francisco and the University of Pittsburgh.

3.3.2. Serological testing and definition of algorithm for defining KSHV seropositivity

Antibody testing was performed using enzyme-linked immunosorbent assays (ELISA) based on peptides from the open reading frames (ORF) 65 and K8.1 with sequences;

ASDILTTLSSTTETAAPAVADARKPPSGKKK and RSHLGFWQEGWSGQVYQDWLGRMNCSYENMT

respectively as previously described (329, 400) with modifications. In brief, serum at a dilution of 1:100, was added to 4 wells: two coated with peptide (5 µg/mL) and the remaining 2 wells without peptide. Optical density (OD) values were read on a Dynatech Laboratories (Chantilly, VA) MRX 1CXA0716 plate reader at 405nm wavelength after reaction with rabbit anti-human IgG HRP (1:6000, DAKO, Carpinteria, CA) and development in 3,3',5,5'-tetramethylbenzidine (BioRad, Hercules, CA) . ODs for a given sample were calculated as the mean OD of the wells containing peptide minus the mean OD of the wells containing no peptide.

The assay cutoff for each peptide was determined a priori to be 0.04 based upon receiver operator characteristic curve analyses. Specimens were considered positive if the OD of both ELISA assays was greater than 0.04, negative if both assays had ODs less than or equal to 0.04 and equivocal if the two assays were discordant. Concordance for the ELISA assays was 96% (Figure 3-1) and all internal controls were correctly identified by both assays. Samples with discordant ELISA results (positive for one antigen and negative for the other) were retested using an indirect immunofluorescence assay (IFA) as previously described.(174) Patients reactive in 2 of the 3 seroassays were classified as KSHV seropositive. The algorithm for defining seropositivity was determined to be 99% specific and 88% sensitive based upon quality control panels (data not shown). To elucidate the relationship between KSHV and PPH and secondary PH, the following statistical tests and measures of association were utilized where appropriate; Fisher's exact test, Student's t-tests, exposure odds ratios, and exact 95% confidence limits.

3.4. Results

Cases and controls were similar with respect to age and gender; and at time of enrollment, patients with PPH and secondary PH had similar pulmonary artery pressures $P=0.35$ (table 4-1).

Table 3-1. Demographic and clinical characteristics of study subjects.

Characteristic	PPH (N=19)		Secondary PH (N=29)		Controls (N=150)
	Number(%)	p-val	Number(%)	p-val	Number(%)
Gender					
Male	5 (26.3)		12 (41.4)		54 (36.0)
Female	14 (73.7)	0.29 [†]	17 (58.6)	0.36 [†]	96 (64.0)
Ethnicity					
White	11 (57.9)		13 (44.8)		127 (84.7)
Black	2 (10.5)	0.01 ^{†*}	3 (6.9)	<.001 ^{†*}	2 (1.3)
Hispanic	3 (15.8)		9 (31.0)		8 (5.3)
Asian	3 (15.8)		4 (17.2)		13 (8.7)
	Mean ± sd		Mean ± sd		Mean ± sd
Age	47.4±15.5	0.44 [‡]	50.9±10.1	0.69 [‡]	49.9±13.1
Mean PA pressure (mmHg)	46.4±14.5		42.4±14.0	0.35 ^{‡*}	----

Note: P-values calculated using controls as referent group. PPH, primary pulmonary hypertension; PA, pulmonary arterial; [†]Fisher's exact one-sided p-value; ^{*}P-values calculated as "White" versus "non-White"; [‡]Student's T-Test, pooled variance estimator. [‡]Comparison is PPH versus secondary PH, PA pressure not available for control subjects.

The overall seroprevalence of KSHV among all study participants was 2.0%. The rate among controls was 0.7% (1/150), and among the study participants with PPH we found no evidence of KSHV infection (0/19) (Table 3-2). Of the 29 subjects diagnosed with secondary PH, 3 (10.3%) were KSHV seropositive (2 with PAH secondary to HIV infection and 1 with scleroderma).

There was no significant difference between the observed seroprevalence of KSHV among subjects with PPH compared to controls ($P=0.89$) or patients with secondary PH ($P=0.21$). A significant KSHV association was present between subjects with secondary PH and controls ($P=0.01$) with an odds ratio of 17.3 and 95% confidence interval of 1.3-908.

Table 3-2. Subjects seropositive for Kaposi's sarcoma-associated herpesvirus by assay

Subjects	Number	K8.1 positive	ORF65 positive	KSHV positive
Experimental Controls				
Positive Controls	10	10 (100)	10 (100)	10 (100)
Negative Controls	10	0 (0)	0 (0)	0 (0)
Study Subjects				
PPH	19	1 (5.3)	0 (0)	0 (0)
Secondary PH	29	5 (17.2)	3 (10.3)	3 (10.3)
Controls	150	4 (2.7)	2 (1.3)	1 (0.7)
Secondary-PH Associated Condition				
• Collagen vascular disease	12	2(16.7)	1 (8.3)	1 (8.3)
• Congenital systemic-to-pulmonary shunts	2	0 (0.0)	0 (0.0)	0 (0.0)
• Portal hypertension	4	0 (0.0)	0 (0.0)	0 (0.0)
• HIV infection	4	2(50.0)	2(50.0)	2(50.0)
• COPD	1	0 (0.0)	0 (0.0)	0 (0.0)
• Interstitial lung disease	4	1 (0.3)	0 (0.0)	0 (0.0)
• Sarcoidosis	2	0 (0.0)	0 (0.0)	0 (0.0)

Note: Data presented as no. (%). PPH, primary pulmonary hypertension; PH, secondary pulmonary hypertension; HIV, human immunodeficiency virus; COPD, chronic obstructive pulmonary disease

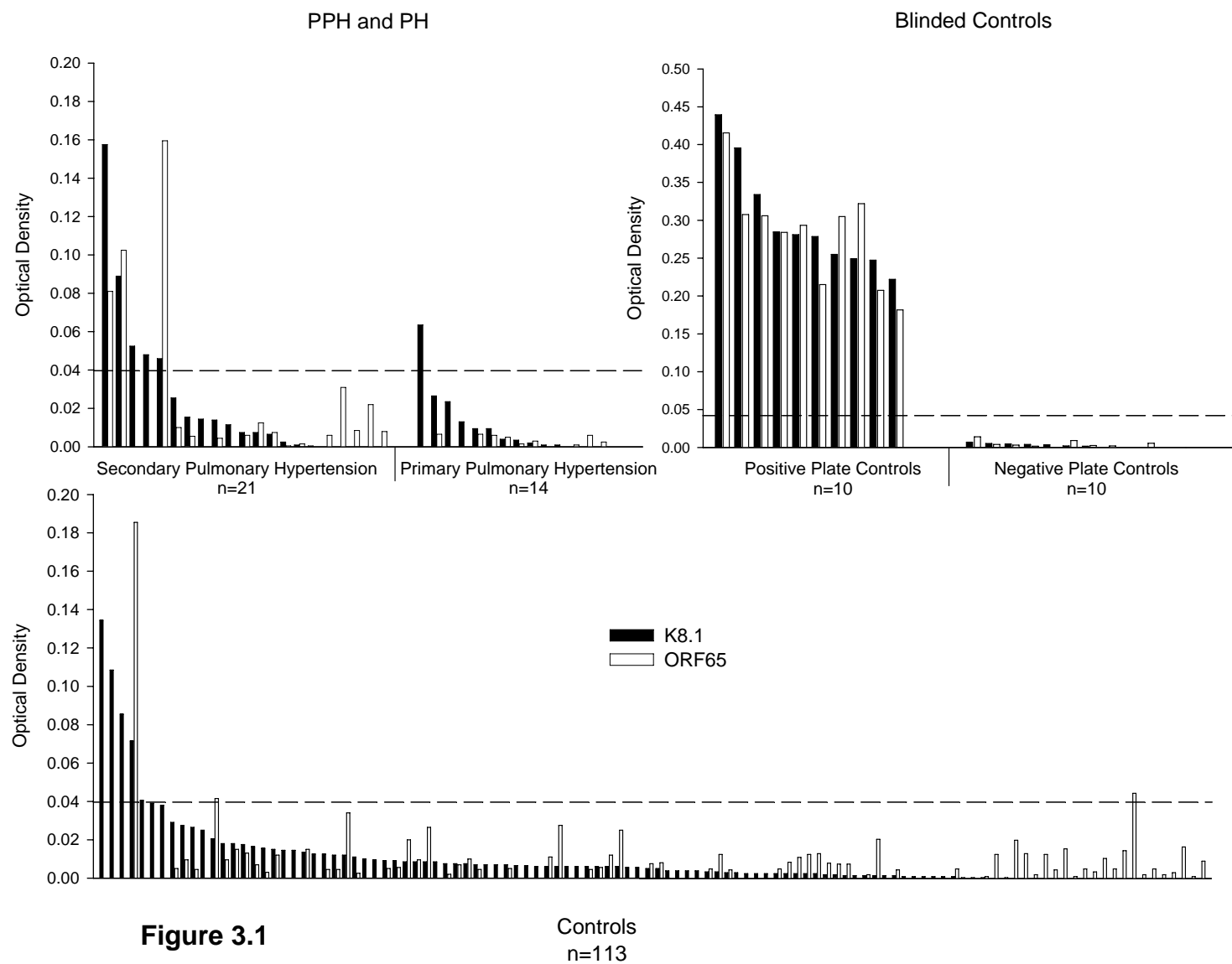


Figure Legend

Figure 3-1. Optical density (OD) values from patients with primary and secondary pulmonary hypertension and controls. Dashed line indicates OD cut-off (0.04) for defining seropositivity. A total of 51 specimens (5 primary PH, 9 secondary PH and 37 controls) with OD values less than zero for K8.1 and ORF65 not represented.

3.5. Discussion

We found no association between KSHV and PPH. Patients with secondary PH had elevated KSHV seroprevalence compared to healthy controls (10.3% vs. 0.7%); an observation which has been previously found among patients with secondary PH (11.8%) and blood donor controls (2.7%).(193) The present findings, of no association between KSHV and PPH (0/19 PPH patients were KSHV seropositive) are in contrast to those of Cool et al.(110) who reported a positive association between KSHV and PPH. Using immunohistochemical staining (IHC) for LANA1, and a polymerase-chain-reaction (PCR) assay on lung DNA to detect the KSHV viral cyclin gene, they found 63% of PPH patients were KSHV infected. In additional contrast, we observed an association between secondary PH and KSHV ($P=0.01$) whereas Cool et al. found no evidence of infection among patients with secondary PH using IHC (1 patient (6.3%) tested positive by PCR). Cool et al. did not include a control group without pulmonary hypertension. However, using the findings from their patients with secondary PH as a comparison group, their data suggest the odds of KSHV infection among patients with PPH is increased over 23 times that of patients with secondary PH (OR=23.3, 95% CI 2.2-1082), a finding we could not replicate with our serological methods (OR=0, 95% CI 0.0, 3.7).

These disparate results may have several explanations. Some of the difference may be explained by different methods used to establish KSHV infection. The techniques, PCR and immunohistochemistry, used by Cool et al. are prone to false-positive reactions in epidemiologic studies and have contributed in the past to spurious associations between KSHV and multiple myeloma,(360, 395) various skin cancers(352) and sarcoidosis.(129) In their reported sequencing of PCR products generated from PPH lesions (see ref (110), Figure 3), all of the products are either too short or too long to be actual amplification products from the

virus and show far higher sequence variation than previously reported--indicating either the detection of novel herpesviruses or that some of these cases are falsely positive. Similarly, special care is needed for immunostaining glandular and epithelial tissues since edge-artifact can be mistakenly interpreted for positivity. It is possible that a similar problem will be encountered with alveolar spaces, which can be easily determined through use of blinded testing of tissues using appropriate control antibodies.

Although it is conceivable that any or all of these issues played a role in the differing results, an alternative to our explanation, for why the present results differ from those previously reported, has already been put forth. Cool et al. have suggested that KSHV serologic tests cannot detect organ-specific infection,(193) which we feel is particularly implausible given the high degree of vascularity present in the plexiform lesions characteristic of pulmonary hypertension, and the lack of evidence for the lung as an immunologically privileged site. Furthermore, KSHV is a gamma-2 herpesvirus that is resident as a lifelong infection of circulating B cells.

Our findings of no association between KSHV and PPH are robust for several reasons: first, the known epidemiology of KSHV is inconsistent with the known epidemiology of PPH. In the United States, KSHV infection is most common in HIV-infected homosexual males; however PPH is significantly more common among young, otherwise healthy, females. KSHV is more common in Mediterranean and African populations and rare in many Asian populations. One would expect the risk group, demographic and geographic distributions of KSHV and PPH to be similar but there is no evidence that this is the case. Second, serological detection of KSHV infection has the advantage that past and current infection can be established irrespective of the site of infection. In addition, properly established serological

assays that have been developed for KSHV since 1998 are less prone to false positive results than other methods such as IHC and PCR. In fact, estimates of KSHV infection rates based on PCR testing among non-Kaposi's sarcoma populations widely vary and range from less than 1 percent to greater than 90 percent(299) and determinations of KSHV infection using IHC are often subjective and prone to non-specific binding which can lead to misdiagnosis of uninfected individuals. Third, our study was of sufficient size to detect a significant effect of KSHV between patients with PPH and controls even if KSHV infection is only associated with one-third of patients with PPH, a rate far below that reported by Cool et al. In sum, our findings of no association between KSHV infection and PPH are robust and are likely generalizable to patients with PPH in the United States.

Though we found no evidence of an association between KSHV seropositivity and PPH, we did observe a correlation between secondary PH and KSHV infection. This may reflect a causal link between KSHV and secondary PH, but it is also likely that these results simply reflect the high rate of KSHV infection among HIV positive individuals. For example, Between 26 and 48% of homosexual men with HIV infection in the US are KSHV seropositive.(275, 320) Because our study was not specifically designed to address HIV-related secondary PH, which requires testing of appropriate HIV-positive controls, we are unable to distinguish among these possibilities. However, if the HIV associated cases are excluded from analysis, the rate of KSHV seroprevalence among the secondary PH group (3.7%) is remarkably similar to that observed in U.S. blood donors (3.3%)(333) and does not significantly differ from that observed in the controls (Fisher $P=0.27$) or in those with PPH (Fisher $P=0.58$).

In conclusion, we find no association between KSHV and PPH and that at most KSHV may be an uncommon contributor to secondary PH. Further, we find no association between KSHV and non-HIV associated secondary PH. We are unable to exclude the possibility that HIV-associated pulmonary hypertension is associated with KSHV but our observations strongly suggest that KSHV does not play an etiologic role in PPH. Furthermore, our findings support that patients with secondary PH or PPH do not have an increased susceptibility to KSHV infection compared to healthy controls.

Addendum: Note that the serological algorithm used for this study of pulmonary hypertension differs from that described in chapter 2.

4. General Discussion

The following section presents a discussion on the subjects of KSHV and the epidemiologic and clinical consequences of misclassification of KSHV infection status and the public health problems associated with viral infection. It is provided as background material not represented as part of the manuscripts or introduction presented above.

4.1. Epidemiologic Consequences of Misclassification of KSHV Infection Status

Though a discussion has begun as to the importance of KSHV surveillance among immunocompromised hosts, the current diagnostic methods described to date have not been adopted for routine KSHV testing(273, 286, 296). This is likely because of the significant misclassification of exposure associated with the currently available assay systems. In a review by Dukers and Rezza, the authors correctly point out that in low prevalence populations most of the currently available assay systems would have a low positive predictive value and a high false positivity rate(142). Specifically, an assay system with sensitivity of 80% and specificity of 99% would produce a positive predictive value of 60.6% and a false positivity rate of 39.4% in a population with an actual KSHV prevalence of 2.5%.

Table 4-1 presents the effect of different assay sensitivities and specificities in low, medium and high KSHV prevalence populations. These values demonstrate the potential for relatively high rates of false KSHV positivity in low prevalence populations with assays of low sensitivity. In KSHV endemic populations (KSHV prevalence of >25%) the false positive rates would decrease closer to acceptable levels, however for most of the developed world KSHV is not a ubiquitous infection. This demonstrates that for screening of populations at increased risk of KSHV morbidities, but low risk of KSHV infection such as transplant donors and recipients, an assay with 80% sensitivity is clearly inadequate as a diagnostic tool for KSHV

prevention efforts. For prevention efforts to be of greatest utility prophylactic treatments and enhanced clinical monitoring must be targeted to those known to be infected. For this goal to be met accurate diagnosis of viral infection must be achieved.

Table 4-1. Variation in predictive values and false positive and negative values in various KS prevalence settings with different assay sensitivities						
Population Prevalence	Assay Sensitivity	Assay Specificity	Positive Predictive Value	Negative Predictive Value	False Positivity Rate	False Negativity Rate
2.5%	85%	95%	30.4	99.6	69.6	0.40
	90%	90%	18.8	99.7	81.3	0.28
	95%	85%	14	99.8	86	0.15
	95%	95%	32.8	99.9	67.2	0.13
15.0%	85%	95%	75	97.3	25	2.7
	90%	90%	61.4	98.1	38.6	1.9
	95%	85%	52.8	99	47.2	1
	95%	95%	77	99.1	23.0	0.9
35.0%	85%	95%	90.2	92.2	9.8	7.8
	90%	90%	82.9	94.4	17.1	5.6
	95%	85%	77.3	96.9	22.7	3.1
	95%	95%	91.1	97.2	8.9	2.8

Note: Predictive values calculated using Bayes theorem(164, 183, 453) where

$$PVP = \frac{(Sensitivity)(Prevalence)}{(Sensitivity)(Prevalence) + (1 - Specificity)(1 - Prevalence)}$$

$$1 - PVN = \frac{(1 - Sensitivity)(Prevalence)}{(1 - Sensitivity)(Prevalence) + (Specificity)(1 - Prevalence)}$$

4.2 KSHV Related Morbidity and Mortality as a Public Health Problem

In the West, among patients infected with KSHV who are immunosuppressed, the risk of developing Kaposi's sarcoma is currently 20%-30%. With a KSHV prevalence of 3%-10% among iatrogenically immunosuppressed patients and 20%-50% among HIV-infected individuals, KSHV is clearly of significant public health concern in the United States, and even

more so in regions with endemic rates of KSHV infection. Of additional concern is that because KSHV is not screened for in the US bloods supply, an estimated 5,000 infections per year via blood transfusions are possible.

It is likely that all of the KSHV-associated morbidity and mortality have not been fully elucidated. For example, the risk of KSHV-related bone marrow failure after transplantation is not well understood and may be more common than is currently thought. Multiple laboratories continue research on KSHV infection in relation to idiopathic conditions. However, screening studies and long-term follow up studies using accurate KSHV detection methods will be the next step in fully understanding the complete extent of KSHV-associated sequelae.

5. Summary

The accurate diagnosis of KSHV infection in individuals is an ongoing challenge for researchers. The body of work in the preceding chapters has described the importance of KSHV seroassays and has illustrated the novel research techniques used to increase diagnostic accuracy. Chapter 2 presented data on creating an assay algorithm which increases diagnostic sensitivity and specificity. In addition the optimized algorithm was used to test KSHV seroprevalence among populations of clinical interest. Article 3 continued the research and looked at patients suspected to be at increased risk of KSHV-associated mortality. In sum, the public health importance of accurately assessing KSHV infection in populations has been addressed and research has been presented which describes an improved method which will potentially allow for a rapid and more accurate characterization of KSHV infection in populations who could benefit from a KSHV surveillance system.

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